University of Alberta

Generation and characterization of chemical-sensitive cell cycle checkpoint mutants in Drosophila

by

Elizabeth Ann Silva ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of Master of Science

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Generation and characterization of chemical-sensitive cell cycle checkpoint mutants in Drosophila* in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Genetics.

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ABSTRACT

Progression through the cell cycle consists of a temporally regulated sequence of events that is monitored by signaling cascades called checkpoints; these checkpoints ensure each event is properly completed before allowing the next to begin. Improper function of any of the components of these checkpoints can result in a number of consequences that jeopardize the welfare of an organism, including cancer or death. Using drugs that are known to perturb cell-cycle progression, I have undertaken three separate genetic studies that investigate various cell cycle checkpoints in Drosophila. First, I endeavored to isolate novel regulators of G2-M progression. Second, I generated mutations in the Drosophila homologue of *ATM*, a known regulator of the DNA damage checkpoint. Third, I have sought to gain insight into the mechanisms of action of three previously identified regulators of the replication checkpoint by assessing and quantifying their responses to chemotherapeutic and DNA damaging drugs.

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LIST OF SYMBOLS AND ABBREVIATIONS

♂	male	**mL**	millilitre
		mM	millimolar
⚥	virgin female	**MMS**	methyl methanosulfonate
		n	number
^{0}C	degrees celsius	**nm**	nanometre
9-1-1	Rad9-Rad1-Hus1	**p**	probability
ara-C	cytosine β-D-arabinoside	**PCNA**	proliferating cell nuclear antigen
A-T	ataxia telangiectasia	**PI3Kc**	phosphoinositide-3-kinase-cognate
BrdU	bromodeoxyuridine	**PNS**	peripheral nervous system
CAS	chemical abstract services	**RDS**	radio-resistant DNA systhesis
CNS	central nervous system	**RNR**	ribonucleotide reductase
DNA	deoxyribonucleic acid	**ROS**	reactive oxygen species
IR	ionizing radiation	**SMW**	second mitotic wave
GMR	*glass* promotor	**UAS**	upstream activating sequence
HU	hydroxyurea	**UV**	ultraviolet radiation
MF	morphogenetic furrow	**X^2**	chi-squared
µg	microgram		

Chapter 1:

Introduction

All organisms require mechanisms for regulating progression through the cell cycle. Each event in the cell cycle must be coordinated with the next to ensure cell division progresses without incident. For example, DNA replication must be completed before mitosis is initiated, spindles must be properly attached and aligned before sister chromatids can be separated and cellular components have to be properly allocated before cytokinesis. In addition to these regulatory mechanisms, there must also be systems in place to guard against cytotoxic stress and to ensure that damaged DNA is not replicated, which would cause mutations to be propagated onto the next generation. Failure to ensure proper regulation at any one of these levels can result in a number of deleterious consequences, including cancer and cell death.

Initial studies of the regulation of cell cycle progression began in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. However for this portion of the discussion I will focus on the work done in *S. pombe*. Among the first mutants isolated were temperature sensitive *cell division control* (*cdc*) alleles, along with alleles of a gene called *wee1*, that were defective in cell cycle progression at various stages, including DNA synthesis, nuclear division and cell plate formation (Nurse *et al.* 1976; Fantes and Nurse 1977; Thuriaux *et al.* 1978; Nurse and Thuriaux 1980). Many of these genes are now known to play essential roles in normal progression through the cell cycle. Disruption of *cdc2* blocks progression into both S phase and mitosis (Nurse and Bissett 1981); disruption of *cdc25* also results in a pre-mitotic block, however this block can be overcome by loss of *wee1* expression (Fantes 1981), indicating that the functions of these two proteins offset one another. Cdc13 interacts with Cdc2 (Hindley and Phear 1984;

Simanis and Nurse 1986) to form a complex that also oscillates through the cell cycle. Peak Cdc13 expression coincides with peak Cdc2 activity, which occurs just prior to mitosis and is down-regulated at the metaphase to anaphase transition (Moreno *et al.* 1989).

Coincident with the cell division control studies were experiments in Xenopus oocytes that identified maturation promoting factor (MPF) (Masui and Markert 1971), a cytoplasmic factor that is required to initiate ovulation and release from a block at prophase of meiosis I in oogenesis. When the molecular nature of MPF was finally revealed, it turned out to be a complex containing a homologue of *cdc2* (Dunphy *et al.* 1988; Gautier *et al.* 1988). The other component of this complex was CyclinB (Gautier *et al.* 1990), one of a number of highly conserved proteins that appear during meiotic and mitotic cycles (Rosenthal *et al.* 1980) and whose homologues include *cdc13* (Booher and Beach 1987; Hagan *et al.* 1988) (the discovery of the association of Cdc2 with CyclinB prompted a "renaming" of *cdc2* as *cyclin dependent kinase 1*, or *cdk1*). These proteins are synthesized during interphase and degraded at onset of cell division (Evans *et al.* 1983). Their catalytic activities oscillate with the cell cycle in Xenopus oocyte development, as measured by histone H1 kinase activity, and exit from mitosis is dependent on their inactivation and degradation by the ubiquitin pathway (Gautier *et al.* 1989; Glotzer *et al.* 1991).

Activation of Cdc2 occurs through a dephosphorylation event that is absolutely required for the G2/M transition. This de-phosphorylation event is the responsibility of Cdc25; in the natural progression of the cell cycle, regulation of Cdc25 activity, which also oscillates with the cell cycle (Moreno *et al.* 1990) links the completion of DNA replication to mitosis (Enoch and Nurse 1990). It is a tyrosine phosphatase that directly activates Cdc2 by removing the phosphate at tyrosine-15 (Russell and Nurse 1986; Gould *et al.* 1990; Gautier *et al.* 1991; Millar *et al.* 1991; Lee *et al.* 1992). Wee1 and its

paralogue Mik1 are tyrosine kinases (Featherstone and Russell 1991; Lee *et al.* 1994) that are part of pathways that phosphorylate Y15 of Cdc2 (Russell and Nurse 1987; Lundgren *et al.* 1991). *In vitro* studies demonstrated that they are also capable of phosphorylating serine/threonine sites (Parker *et al.* 1992), but this activity has not been conclusively demonstrated *in vivo*. These two kinases act cooperatively to maintain phosphorylation of Cdc2 long enough for S phase to be completed (Lundgren *et al.* 1991), thereby counter-balancing the effects of Cdc25.

Thus the cell cycle is, by nature, a unidirectional series of events in which the initiation of one step relies on the completion of the previous step. Is this because the product of one becomes the substrate for the next, or is there a separate mechanism? To investigate this issue, a number of mutants were generated in which this relationship is uncoupled. Genes that were identified by this means are not required for viability under normal circumstances, nor for progression through an undisrupted cell cycle, but are activated in response to genotoxic stress and ongoing DNA replication, and thus are referred to as checkpoints (Weinert and Hartwell 1989). These checkpoints are the source of the dependence of cell cycle progression on undamaged and completely replicated DNA.

A number of screens for checkpoint mutants were conducted in both *S. cerevisiae* and *S. pombe* (Nasim and Smith 1975; Prakash and Prakash 1977; Weinert and Hartwell 1988; Weinert and Hartwell 1989; Lehmann *et al.* 1991; Enoch *et al.* 1992). These mutants, many of which are called *rad* (*radiation sensitive*) mutants, are extraordinarily sensitive to treatment with ionizing radiation. This sensitivity can usually be traced to either their failure to recognize DNA damage and propagate a signal that arrests the cell cycle, or their inability to repair the damage that has been done (Carr 1995). Since many of the *rad* and *rad*-related genes were isolated from each species simultaneously, their names are not consistent between species and are therefore listed in Table 1-1. For the

sake of simplicity, I will in most cases refer to the *S. pombe* nomenclature throughout this text unless otherwise indicated. When the need for clarity dictates, references to these genes will be accompanied by the designation (Sc) for *S. cerevisiae* or (Sp) for *S. pombe*.

The center of regulation for the G2/M checkpoint in both yeasts is Rad3, a phosphoinositide-3-kinase-like protein (PI3Kc) that is involved in both DNA replication and DNA damage responses. Immediately upstream of this kinase are Rad17, Rad1, Rad9 and Hus1 (the isolation of Hus1 will be discussed later). Rad17 has striking structural similarities to replication factor C and Rad1/Rad9/Hus1 form a complex (9-1-1 complex) similar to that formed by proliferating cell nuclear antigen (PCNA) (Venclovas and Thelen 2000). PCNA is a clamp that is responsible for loading replication factors onto sites of initiation for DNA replication (Waga and Stillman 1998). More recently it has been demonstrated in *S. cerevisiae* that the homologue of Rad17, Rad24 (Sc), loads onto DNA at sites of double strand breaks, and DNA-bound Rad24 (Sc) then recruits the Rad1, Rad9 and Hus1 homologues, Rad17, Ddc1, and Mec3 (Sc), which in turn activate Mec1 (Sc) (Kondo *et al.* 2001).

While *S. cerevisiae* and *S. pombe* have many homologous checkpoint components, they also have many different requirements for regulating the cell-cycle in response to DNA damage and ongoing replication. In *S. cerevisiae*, only the response to DNA damage relies on the proper function of the Rad17 and 9-1-1 complex homologues while incomplete replication triggers the activity of a putative polymerase called Pol2 (Navas *et al.* 1995). However, in *S. pombe* the responses to both DNA damage and incomplete replication rely on the function of Rad17 and the 9-1-1 complex. So, while it is appealing to draw analogies between the mechanisms of action for this complex in *S. pombe* and *S. cerevisiae*, there is not yet evidence for the ability of Rad17 (Sp) to directly recognize ongoing replication, leaving open the possibility that Rad17 (Sp) may have species specific functions.

Further differences are seen in the targets of the Rad3 pathway. In *S. pombe*, the action triggered by Rad3 is determined by the type of DNA damage incurred; a need for S phase arrest results in different consequences than a need to arrest cell cycle in response to DNA damage. In response to incomplete replication, Rad3 initiates a cascade that is propagated through Cds1 and the Wee1 and Mik1 kinases (Murakami and Okayama 1995), culminating in the inhibitory phosphorylation of Cdc2. The response to DNA damage, which also involves wee1 activity (Rhind *et al.* 1997), is propagated through chk1 (Walworth *et al.* 1993) and involves the activation of Rad24, a homologue of the 14-3-3 proteins. 14-3-3 is capable of affecting arrest by sequestering Cdc25 from the nucleus and by affecting its catalytic activity (Lopez-Girona *et al.* 1999; Lopez-Girona *et al.* 2001).

In *S. cerevisiae*, the type of damage also has an effect on the course of action. Activation of the Rad3 homologue, Mec1, triggers the propagation of a signal through Rad53 kinase in response to DNA damage at all stages of the cell cycle, as well as in response to incomplete replication (Weinert *et al.* 1994; Sanchez *et al.* 1996). However, cdc28 is not the target of the Mec1-triggered pathway (Amon *et al.* 1992; Sorger and Murray 1992) and there is no involvement of BMH1, the *S. cerevisiae* homologue of 14-3-3 (Gelperin *et al.* 1995).

While basic machinery for regulating cell cycle progression is well conserved between all eukaryotes (Nurse 1990), there is a limit to the extent that analogies can be drawn between yeast and metazoans. Multicellular organisms have unique requirements in the regulation of cell division. They must have the ability to recognize cellular stress and respond to it in the same way that yeast does, but they must also regulate progression through the cell cycle in other circumstances as well. The process of development involves a number of events that require rearrangements of both cells and tissues, and these events must be coordinated with cell division. Since cell migration and changes in

cell shape and morphology utilize the same basic cellular machinery as cell division, these events must be temporally or spatially separated (Leptin 1995). One of the primary mechanism for doing so is through initiation of cell cycle checkpoints (Grosshans and Wieschaus 2000; Mata *et al.* 2000; Seher and Leptin 2000). Multicellular organisms also have a requirement for tissue and cell-specific regulation. Mitotic divisions in some somatic tissues must be monitored to prevent overgrowth while others need to continue to produce new cells throughout the life span of an organism. Uncontrolled cell division in a vertebrate is defined as cancer, which can threaten the life of the organism. As such, even the homologues of cell cycle components that were isolated in yeast as serving true "checkpoint" functions take on unique roles in metazoans and thus become essential for viability.

Drosophila is the model system that we have chosen for examining cell cycle regulation, since it is one of a few metazoans in common use that has the advantage of being a truly genetically amenable system. Initial studies of cell-cycle regulation in Drosophila focussed on the embryo. After fertilization, Drosophila embryos undergo a series of synchronous nuclear divisions contained within a single syncytium (Foe and Alberts 1983). The first divisions proceed rapidly through S-M type cycles until cycle 11, when there is progressive lengthening of interphase. The timing of these divisions is under the control of a checkpoint pathway that is homologous to the G2-M checkpoint elucidated in *S. pombe*, the Dwee1 (wee1)/grapes (Chk1)/mei-41 (Rad3) pathway (Fogarty *et al.* 1994; Sibon *et al.* 1997; Sibon *et al.* 1999; Price *et al.* 2000). All of the early embryonic events, including these divisions, are maternally controlled (Edgar *et al.* 1986); therefore, zygotic expression of *Dwee1*, *grapes* (*grp*) or *mei-41* is not required under normal growth conditions. Maternal expression of the Drosophila homologues of *cdc25*, *string* (*stg*), and *cdc2/cdk1* are also required for early embryonic development (Edgar and O'Farrell 1989); as in *S. pombe*, Cdc25-stg dependent dephosphorylation of

Cdk1-Y15 is required for entry into mitosis (Stern *et al.* 1993; Edgar *et al.* 1994). Again as in *S. pombe*, CyclinB accumulates just prior to initiation of mitosis and is inactivated at the metaphase to anaphase transition, however Drosophila embryos also require CyclinA activity for mitotic progression, which acts synergistically to CyclinB (Lehner and O'Farrell 1990; Knoblich and Lehner 1993).

These and many other Drosophila checkpoint mutants were isolated based on simple defects in embryonic development. For example, *cdc25-stg* and *grp* were both originally identified based on their requirements for early embryonic development (Jurgens *et al.* 1984; Sullivan *et al.* 1993); *mei-41* was identified based on its requirement for meiosis (Baker and Carpenter 1972). Very labor-intensive studies were undertaken by groups that looked for defects in embryonic development (Grosshans and Wieschaus 2000; Mata *et al.* 2000) and patterns of G1/S progression (Royzman *et al.* 1997; Whittaker *et al.* 2000). These screens resulted in the isolation of novel checkpoint genes such as *tribbles* and *double-parked*, respectively.

Checkpoint function has also been studied extensively in the Drosophila eye, a developmental system that is particularly amenable to such experiments due to its strict requirements for regulation and the fact that any disruption of this regulation is translated into easily recognized phenotypes. The eye tissue is also dispensable for viability of the organism, so mutations that affect checkpoint function in the eye will not necessarily result in death. The adult eye consists of about 800 precisely arranged ommatidia, each of which is comprised of an invariant structure arising from 19 precursor cells (Figure 1-1, A and B). Fourteen of the 19 precursor cells are born during third larval instar development of the primordial eye tissue, the eye imaginal disc. The transition from proliferation to differentiation of these cells is demarcated by the morphogenetic furrow (MF), a dorsal-ventral indentation in the surface of the disc that has the appearance of progression across the disc from posterior to anterior (Figure 1-1C). Ahead of the

furrow, cells divide in an asynchronous manner but when they enter the furrow they undergo a synchronous arrest in G1. Within the furrow, cells are organized into rosettes, each of which constitutes an ommatidial precursor. As the cells emerge all those that have not been recruited into the ommatidial precursors undergo a tight wave of DNA replication that is referred to as the second mitotic wave (SMW), followed by a less synchronous band of mitoses (Wolff and Ready 1991a; Wolff and Ready 1993). In many cells replication is not immediately followed by division. In fact, some cells remain in G2 arrest indefinitely, as is indicated by undiminished levels of cyclin B accumulation (Figure 1-1D) (Baker and Yu 2001). Each of these events is strictly regulated by tissue specific expression of cell cycle surveillance mechanisms. Some checkpoint genes that are required in the Drosophila eye are the same as those that are required in the development of the early embryo, however regulation of cell cycle progression in the eye is further complicated by the need to coordinate cell division with differentiation. Therefore the eye has some unique requirements.

As in the embryo, *cdc25-stg* expression is required for proper progression through mitosis; *Drop*, a cis-regulatory allele of *cdc25-stg* that inactivates expression in the eye, causes cells to experience a premature G2 arrest, accumulating high levels of cyclinB as they enter the MF (Mozer and Easwarachandran 1999). Expression of *roughex (rux)*, a cyclin dependent kinase inhibitor, is necessary for maintenance of G1 arrest in cells that have been committed to the neuronal cell fate. In the absence of *rux*, all cells enter S phase (Thomas *et al.* 1994) due to the ectopic activation of CyclinA/Cdk complexes (Dong *et al.* 1997), which prevent mitotic CyclinB/Cdk1 activity via direct inhibition of Cdk1 (Foley *et al.* 1999). Phenotypes observed in *rux* mutants are suppressed by mutations in eye-specific alleles of *cdc25-stg*. Progression from G2 to M in the second mitotic wave is regulated by signals that are transmitted from the preclusters, through the

epidermal growth factor receptor (EGFR), to surrounding cells resulting in activation of cdc25-stg (Baker and Yu 2001).

Development in the Drosophila eye is also characterized by extensive cell death during pupal development (Wolff and Ready 1991b). At this stage of development, all cells that have not been recruited to a developmental fate undergo apoptosis. Cell division and apoptosis are frequently regulated by similar mechanisms and even require activation of some of the same proteins; induction of apoptosis is an alternative to cell cycle arrest that is frequently employed when cells have sustained too much damage to repair. In the case of the Drosophila eye, the EGFR pathway not only regulates G2/M progression, but is also required for protection from apoptotic cell fates (Miller and Cagan 1998; Baker and Yu 2001; Freeman and Bienz 2001). Another excellent example of this duality is the function of p53. In its response to DNA damage, p53 can direct the induction of either p21-dependent induction of a G1 cell cycle arrest or apoptosis (Vogelstein *et al.* 2000); over-expression of p53 in the Drosophila eye also results in extensive cell death (Jin *et al.* 2000). Numerous other over-expression studies of both apoptotic promoters and inhibitors in the Drosophila eye have revealed that any disruption of the programmed cell death pathways disrupts normal patterning (Hay *et al.* 1994; Hay *et al.* 1997; Kurada and White 1998; Freeman and Bienz 2001). Thus in addition to its suitability as a system for examining cell cycle regulation, the eye is an excellent system for studying DNA damage-triggered apoptosis.

The genetically amenable system of the Drosophila eye tissue as a means of identifying genes involved in cell cycle regulation has been made easier by various over-expression and recombination systems that are available. Many groups have used various combinations of the GAL4-UAS system, in which ectopic expression of the transcriptional activator GAL4 is under the control of a tissue specific promotor. Expressed GAL4 then binds to an upstream activating sequence (UAS) that has been

inserted next to the gene of interest, initiating its transcription (Brand and Perrimon 1993). This system allows for study of overexpression phenotypes in a range of tissues, which in many cases assists in the elucidation of the function of a given gene. Genes can also be put under direct control of tissue specific promotors such as the *glass* promotor (GMR) (Hay *et al.* 1997), which drives expression in the developing eye posterior to the morphogenetic furrow. These constructs can then be used to screen for mutations that suppress or enhance ectopic overexpression phenotypes to identify previously undiscovered genetic interactions. This system has been used to identify genetic interactions with *CyclinE* (Lane *et al.* 2000) as well as the Drosophila Wee1 kinases, *Dwee1* and *Dmyt1* (Price *et al.* 2002). Similar approaches have also been employed in screening for modifiers of loss-of-function phenotypes of eye-specific cell-cycle regulators such as *rux* (Thomas *et al.* 1997). Another useful strategy is mosaic analysis of the eye, which can be done using a site-specific recombination system adapted from yeast. Eye-specific expression of an enzyme called FLPase induces recombination between adjacently located transgenic FRT sites resulting in clonal patches of homozygous and heterozygous eye tissue (Golic and Lindquist 1989; Golic 1991). The FLP-FRT system has the advantage of allowing for identification of genes that are essential for viability but are also involved in tissue specific processes, without compromising the integrity of the organism. The proximity of heterozygous and mutant cells that result from the induction of FLP-mediated recombination also allows for examination of a wide range of phenomena, including cell competition, cell growth, signaling mechanisms and cell adhesion (Xu and Rubin 1993; Walsh and Brown 1998; Moberg *et al.* 2001).

Another method for identifying novel cell-cycle regulators has been through the use of various DNA damaging and cell-cycle perturbing agents. I have already discussed the isolation of the *rad* mutants in yeast, but similar methods have been used in numerous

screens in both Drosophila and yeast, using a range of chemicals. As with the screens for the *rad* mutants, this strategy is based on the assumption that true checkpoint genes are not necessary for viability under normal growth conditions; they are essential only under circumstances of genomic or cellular stress. While the majority of checkpoint components in Drosophila have essential roles in some aspect of development, a few are required strictly for the damage response. The Drosophila *mus* (*mutagen-sensitive*) mutants were originally isolated based on their sensitivities to DNA damage resulting from exposure to ionizing radiation (Boyd and Setlow 1976; Brown and Boyd 1981). The majority of these genes have since been implicated in DNA repair pathways, but characterization of mus304 has more recently identified it to be a novel DNA damage checkpoint gene (Brodsky *et al.* 2000). A mammalian homologue of *mus304*, *ATRIP*, has also been isolated based on its ability to interact with the mammalian homologue of *mei-41*, *ATR* (Cortez *et al.* 2001).

Even those checkpoint components that are required during development often serve dual roles in maintaining genome fidelity while ensuring proper coordination of developmental events. One chemical that is commonly used to investigate checkpoint mutants for putative roles in the response to DNA damage is methyl methanosulfonate (MMS), a mutagen that acts through DNA alkylation. In addition to their sensitivities to various types of ionizing radiation, many of the *rad* mutants are sensitive to exposure to MMS. While it is true that a maternal contribution of Drosophila *mei-41* and *grp* is required for early development, zygotic expression of these genes is not required under normal growth conditions. However, zygotic functions of mei-41 and grp are required when developing organisms are exposed to MMS, indicating that these checkpoint components have essential roles in the recognition and response to DNA damage. (Baker *et al.* 1976; Mason *et al.* 1981; Banga *et al.* 1986; Radcliffe *et al.* 2002). These data emphasize the notion that many metazoan checkpoints are not simply surveillance

mechanisms, nor do they serve strictly developmental roles. On the contrary, they are required both for maintaining genome fidelity while ensuring proper coordination of developmental events.

One particularly noteworthy drug that has been used to assay checkpoint function is hydroxyurea (HU). HU inhibits ribonucleotide reductase, an enzyme required for the generation of deoxyribonucleotides. In the presence of low doses of HU, cells with intact G2-M checkpoints will initiate a delay that is sufficient to ensure proper DNA replication before the initiation of mitosis. Mutants for *rad3*, *wee1* and *cds1*, as well as their Drosophila counterparts *mei-41*, *Dwee1* and *grp*, are sensitive to HU exposure (Banga *et al.* 1986; Enoch *et al.* 1992; Fogarty *et al.* 1994; Sibon *et al.* 1997; Boddy *et al.* 1998; Price *et al.* 2000). Presumably these mutants die due to their inability to initiate the appropriate checkpoint and consequently proceed prematurely through cell division. The *hus* (*hydroxyurea-sensitive*) mutants in *S. pombe*, as the name suggests, were identified based on their sensitivities to HU (Enoch *et al.* 1992). Recall that Hus1 is a participant in the G2/M checkpoint that functions upstream of Rad3 via its interactions with Rad1, Rad9 and Rad17.

Exposure to caffeine causes over-ride of an HU-induced cell-cycle arrest (Powell *et al.* 1995; Wang *et al.* 1999) by general interference with the kinase functions of the phosphoinositide-3-kinases (Sarkaria *et al.* 1999; Moser *et al.* 2000). In fact the checkpoint pathways that involve Rad3 and its homologues are frequently referred to as the caffeine sensitive pathways. Although caffeine has been widely used for its checkpoint perturbing functions *in vitro*, it has only recently been employed for *in vivo* analysis of checkpoint mutants. In fact, only a few studies in yeast have looked at altered caffeine sensitivity as a means of analyzing checkpoint pathways (Benko *et al.* 1997; Rowley and Zhang 1999; Arudchandran *et al.* 2000). However, functional redundancies are common in the regulation of the cell cycle and, evolutionarily speaking, it is

reasonable to suppose that natural selection would favor the generation of back-up mechanisms to ensure that failure of one component of one checkpoint does not necessarily result in lethality of the entire organism. As such, studies are emerging in which checkpoint mutants are isolated based on sensitivities to checkpoint-inducing chemicals that are compounded by caffeine, with the rationale that inhibition of the PI3Kc proteins might make mutations in other pathways more apparent (Wang *et al.* 2000).

Sensitivity of checkpoint mutants to disruption by cell cycle perturbing drugs is the basis for the rationale behind chemotherapy, since cells isolated form a wide range of tumor types all have defects in one or more components of the checkpoint pathways (Levine *et al.* 1991; Futreal *et al.* 1994; Bay *et al.* 1999). Chemotherapeutic drugs, which include nucleoside analogues and DNA damaging agents, are utilized for their abilities to target rapidly dividing cells such as those found in cancerous tissue, thus drugs that target checkpoint defective cells are continually being developed and tested in mammalian systems.

Study of checkpoint mechanisms in metazoans is complicated by the existence of complex regulatory systems that involve multiple pathways that respond to DNA damage, where the nature of the response depends on the stage of the cell cycle and the type of damage that is induced. Drosophila has three documented PI3Kc proteins, humans have at least six, some of which are listed in Table 1-1, and so far evidence indicates that each is capable of triggering a distinct cascade in response to a variety of cellular cues. The canonical G2/M checkpoint pathway that requires mei-41 in Drosophila and ATR in vertebrates is triggered in response to both incomplete replication and DNA damage, but only if the damage is incurred during S or G2 phase (Hall-Jackson *et al.* 1999; Liu *et al.* 2000; Zhao and Piwnica-Worms 2001). DNA damage caused by ionizing radiation during G1 or just prior to S phase results in initiation of the ATM-

dependent pathways, and does not involve ATR/mei-41 (Canman *et al.* 1998). This pathway involves inhibitory phosphorylation of cdc25 in a manner dependent on Chk2, a homologue of *cds1* (Chaturvedi *et al.* 1999; Falck *et al.* 2001; Xu *et al.* 2001).

The ATM-dependent pathways have been extensively studied due to the implication of mutations in numerous components of these pathways in tumorogenesis and pre-disposition to cancer. Among ATM's many targets are breast cancer antigen 1 (BRCA1), the tumor suppressor p53 and the protein that is defective in Nijmegen Breakage Syndrome, Nbs1 (Canman *et al.* 1998; Li *et al.* 2000; Lim *et al.* 2000; Zhao *et al.* 2000). The intricacies of these pathways require far more attention than it is possible to give here, but some of the many functions of ATM are discussed in Chapter 2. Another distinct checkpoint cascade that responds to DNA damage requires no involvement of the PI3 kinases. UV damage, depending on the stage of the cell cycle, initiates a cascade that differs from the one initiated in response to ionizing radiation; studies in mammalian systems have implicated a caffeine-insensitive pathway in this response (Goldstone *et al.* 2001). This pathway involves the activation of a mitogen-activated protein kinase called p38a (Bulavin *et al.* 2001), which is able to inactivate Cdc25 in response to UV, just as Chk1/grp inactivates cdc25 in response to incomplete replication.

Multi-cellular organisms are also able to employ induction of apoptosis in their responses to DNA damage as a way of sacrificing the few to save the whole. I have already briefly mentioned the dual roles of p53 in apoptosis and cell cycle arrest; in fact, p53 is the central participant in this mechanism. Among the many unique roles p53 plays in the intricacies of mammalian cell cycle regulation are: its activation via the ATM-mediated pathways in response to ionizing radiation (Carr 2000); its activation via the ATR-mediated pathways in response to UV, various chemotherapeutic drugs and protein kinase inhibitors (Meek 1999); and its ability to direct both cell cycle arrest and apoptosis

(Kuerbitz *et al.* 1992; Chiarugi *et al.* 1994; Gottlieb and Oren 1998). Its activity is abrogated in most cancers and thus has received a great deal of attention over the last thirteen years (Levine 1989). However, despite the intense amount of research that has been conducted, there are still many unanswered questions, particularly regarding the mechanisms that direct the mode of action of p53.

My work has focussed on checkpoint mutants and their responses to cell cycle perturbation by various chemical means. The first of my studies was a screen for mutants whose phenotypes are sensitive to hydroxyurea and caffeine using an elegant system devised by Stowers and Schwarz (1999) as a means of identifying genes that are essential for eye development. Analysis of the mutants isolated from this screen focussed on two loci: the first is an essential gene, the second is essential only in the presence of caffeine. I have not yet been able to conclusively demonstrate a role for these genes in checkpoint function, but the mutant phenotypes in and of themselves are of great interest.

My second study is fairly straightforward: the generation of mutations in Drosophila *ATM*. This study involves a basic F2 deficiency screen, in which I isolated alleles for *dATM* by scoring for lethality and sterility both in the absence and presence of caffeine. Based on the visible phenotypes that are observed, I also hope to develop this system as a model for the study of human ataxia telangiectasia, a disease that manifests as the result of mutations in ATM.

My third and final study involved the examination of three Drosophila checkpoint mutants, *mei-41*, *grp* and *Dwee1*, in their sensitivities to various DNA damaging and chemotherapeutic drugs. Through this study, sensitivities were quantified and statistically verified with two purposes in mind. The first was to gain some insight into the complex intricacies of the DNA damage and DNA replication checkpoints. The second was to investigate the feasibility of using Drosophila checkpoint mutants as an assay for the effects of novel chemotherapeutic agents.

Table 1-1. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* cell cycle genes and their homologues[a].

S. cerevisiae	*S. pombe*	Drosophila	mammalian	structure and/or function
Rad24	Rad17	Rad17	hRad17	RF-C like DNA-binding protein
Rad17	Rad1	Rad1	hRad1	component of the 9-1-1 complex
Ddc1	Rad9	Rad9	hRad9	component of the 9-1-1 complex
Mec3	Hus1	Hus1	hHus1	component of the 9-1-1 complex
Mec1 Tel1	Rad3 Tel1	mei-41 dATM	ATR ATM TRRAP DNA-PK ATX	components of the PI3Kc family that respond to various cellular cues including cell growth and genotoxic damage
Chk1	Cds1	grp/Chk1	Chk1	G2/M checkpoint kinases
Rad53	Chk1	Chk2	Chk2	G1/S checkpoint kinases
Swe1	Wee1 Mik1	Dwee1 Dmyt1	Hu-Wee1 Hu-Myt1	checkpoint kinases that phosphorylate Cdc2
?	Cdc25	string	Cdc25A Cdc25B Cdc25C	Cdk-specific phosphatases
Cdc28	Cdc2	Cdk1 Cdk2	Cdk1 Cdk2	Cyclin-dependent kinases
Cln1,2,3 Clb1-5	Cdc13	CyclinB CyclinA	CyclinB CyclinA	Cyclins
BMH1	14-3-3	14-3-3	14-3-3	inhibitors of Cdc25

a. Only those cell-cycle regulators that are included in the discussion are listed here.

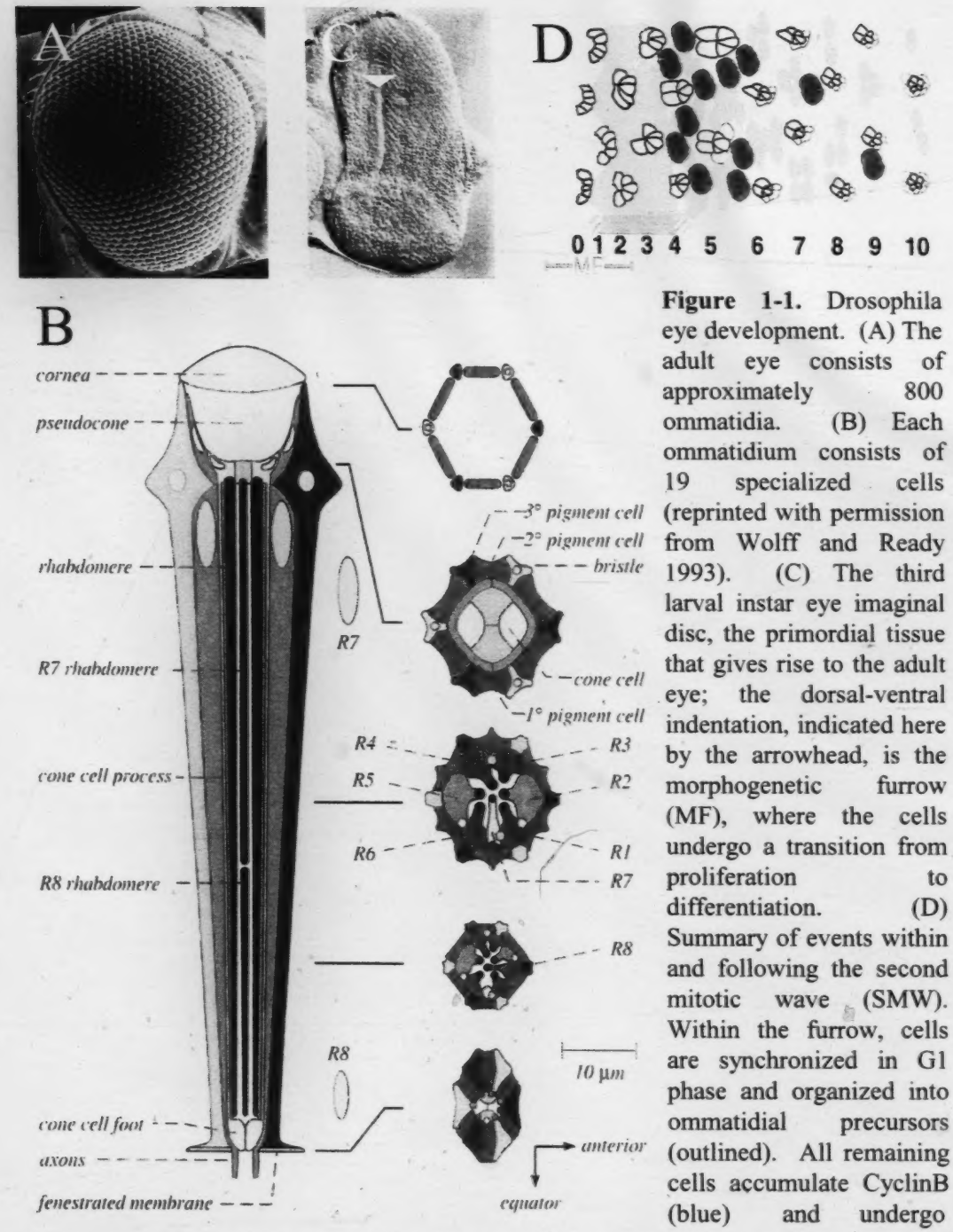

Figure 1-1. Drosophila eye development. (A) The adult eye consists of approximately 800 ommatidia. (B) Each ommatidium consists of 19 specialized cells (reprinted with permission from Wolff and Ready 1993). (C) The third larval instar eye imaginal disc, the primordial tissue that gives rise to the adult eye; the dorsal-ventral indentation, indicated here by the arrowhead, is the morphogenetic furrow (MF), where the cells undergo a transition from proliferation to differentiation. (D) Summary of events within and following the second mitotic wave (SMW). Within the furrow, cells are synchronized in G1 phase and organized into ommatidial precursors (outlined). All remaining cells accumulate CyclinB (blue) and undergo synchronous S phase in the SMW (marked by the gold bar), followed by less synchronous mitoses (red). Some cells never divide, retaining CyclinB accumulation indefinitely (reprinted with permission from Baker and Yu 2001).

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Chapter 2:

A method for assaying the sensitivity of Drosophila replication checkpoint mutants to anti-cancer and DNA damaging drugs.

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INTRODUCTION

Eukaryotic organisms utilize signaling pathways called "checkpoints" that ensure proper coordination of cellular events throughout the cell cycle. Organisms with deficiencies in these checkpoints are sensitive to a variety of DNA damage agents and to chemicals that perturb DNA replication, including various anti-cancer agents that are utilized for their ability to preferentially disrupt rapidly dividing cells.

The sensitivity of checkpoint mutants to DNA damaging chemicals has been widely documented. In *Schizosaccharomyces pombe*, a number of mutants that were originally isolated as mutagen-sensitive have since been implicated for involvement in both DNA replication and DNA damage checkpoints; these include the *hus* (hydroxyurea sensitive) and *rad* (radiation sensitive) mutants (Lehmann *et al.* 1991; Enoch *et al.* 1992; Carr 1995). These genes encode components of a family of evolutionarily conserved kinases, whose activities link responses to DNA damage and incomplete DNA replication with regulators of the cell cycle and other cellular responses, notably DNA repair. In *S. pombe*, for example, incomplete DNA replication triggers activation of Rad3, a homologue the ATM/ATR/mei-41 kinases found in humans and Drosophila, which in turn activates checkpoint kinases Chk1 and Cds1/Chk2. Chk1/Chk2 initiate a block in

mitosis by inhibiting the mitotic regulator Cdk1 via inhibitory phosphorylation, through simultaneous promotion of Cdk1-inhibitory Wee1 kinase activity and inactivation of the Cdk1 activator, Cdc25 phosphatase (Furnari *et al.* 1997; Boddy *et al.* 1998; Rhind and Russell 1998a; Rhind and Russell 1998b; Brondello *et al.* 1999; Furnari *et al.* 1999). These mutants, as well as mutants for the *Drosophila* components of this checkpoint pathway, *mei-41* (*ATR*), *grp* (*Chk1*) and *Dwee1* (*Wee1*), are all sensitive to hydroxyurea (HU), a chemical that inhibits ribonucleotide reductase (RNR) and thereby disrupts DNA replication (Banga *et al.* 1986; Lundgren *et al.* 1991; Enoch *et al.* 1992; Fogarty *et al.* 1994; Sibon *et al.* 1997; Rhind and Russell 1998a; Sibon *et al.* 1999; Moser and Russell 2000; Price *et al.* 2000).

Direct screens for *Drosophila* mutagen-sensitive mutants based on their sensitivity to ionizing radiation identified a number of different *mus* genes, many of which are sensitive to multiple types of DNA damage (Boyd and Setlow 1976). Most of the *mus* genes that have been molecularly characterized encode components of DNA repair pathways but retain functional cell cycle checkpoints (Brodsky *et al.* 2000). In contrast, the *mus304* gene encodes a novel DNA damage checkpoint protein and *mus304* mutants exhibit a similar maternal lethal phenotype as mutations in the *Dwee1*, *grp* and *mei-41*, the checkpoint genes that are characterized in this report (Henderson 1999; Brodsky *et al.* 2000). A human homolog of *mus304* (ATRIP) has recently been identified by its physical interaction with human ATR, the functional homolog of *mei-41*, suggesting that mus304/ATRIP might function as a co-factor or perhaps as a scaffold protein for ATR checkpoint signaling events (Cortez *et al.* 2001).

Human cell lines isolated from various types of tumors have been found to be deficient in one or more components of the pre-mitotic DNA replication and DNA damage checkpoints, suggesting that loss of function for such genes is a common step in tumorigenesis. Mutations in any of *p53*, *ATM*, *Chk2*, *Cdc25*, *BRCA1* or *BRCA2*, whose

normal functions are required to properly regulate Cdk1 by inhibitory phosphorylation, result in genome instability and increased susceptibility to various types of cancers (Levine *et al.* 1991; Kastan *et al.* 1992; Kuerbitz *et al.* 1992; Futreal *et al.* 1994; Savitsky *et al.* 1995; Bay *et al.* 1999; Cortez *et al.* 1999; Zhou and Elledge 2000; Davies *et al.* 2001). Transformed cells that are mutant for checkpoint functions are more susceptible to DNA damaging agents than non-proliferating cells with intact checkpoints, allowing them to be preferentially destroyed.

In developing potential DNA damage and anti-cancer agents, it would be useful to have a quick and direct method for assessing their effects on an intact multi-cellular organism. Such an assay would facilitate the effort to identify compounds that are capable of specifically targeting cells with compromised checkpoints, with minimal detriment to surrounding tissue. Here we investigate the sensitivity of three previously characterized *Drosophila* checkpoint mutants (*mei-41*, *grp* and *Dwee1*) to five chemicals that are known to either damage DNA or delay DNA replication. The zygotic function of each of these genes is not essential for viability under normal growth conditions. However, previous studies have shown that when checkpoint mutant larvae are grown under conditions where DNA replication is partially disrupted by HU, the mutant larvae die at doses that wild-type siblings tolerate reasonably well, presumably because the mutants are unable to initiate a cell cycle delay in response to incomplete replication. These mutants attempt to undergo mitosis before replication is complete, which is a lethal event. We now report that these three checkpoint mutants exhibit markedly different sensitivities to hydroxyurea as well as to the DNA damaging agents methyl methanosulfonate (MMS), ara-C, cis-platin and an oxygen radical-generating compound, methyl viologen (paraquat). Three of these chemicals, HU, ara-C and cisplatin, are currently used for chemotherapeutic treatment of cancer. In statistically relevant comparisons, we have found a surprising diversity of responses among the different

mutants, suggesting that requirements for individual components of the Mei-41/Grp/Wee1 checkpoint pathway are distinct for responses to different chemicals. Information of this sort derived from fly models could potentially be used to design 'customized' chemotherapy regimes for individuals whose tumor cell checkpoint gene status is known, pin-pointing those chemicals that can effectively target cells with a particular checkpoint defect.

MATERIALS AND METHODS

Stocks and crosses performed: Stocks utilized in this analysis were: *w; Dwee1^{ES1}/CyO P{act-GFP, w$^+$}, w; Dwee1^{W05} P{act-GFP, w$^+$}, w; grp-1/CyO P{act-GFP, w$^+$} and w; mei-41^{D3}/FM7 P{act-GFP, w$^+$}*. *Dwee1^{ES1}* and *Dwee1^{W05}* were previously described by Price *et al.* (2000), *grp-1* was previously described by Bier *et al.* (1989) and Fogarty *et al.* (1994), *mei-41^{D3}* was previously described by Banga *et al.* (1995), Hari *et al.* (1995) and Sibon *et al.* (1999). For analysis of *Dwee1* mutants, *Dwee1^{ES1}* females were mated to *Dwee1^{W05}* males; for analysis of *grp-1* and *mei-41^{D3}* mutants, male and female heterozygotes were mated together. After mating, females were allowed to deposit eggs on standard molasses and cornmeal media over a 24 hour interval, after which the brood was allowed to develop for a further 24 hours. The media was then stabbed with a clean metal spatula and stock solutions of each chemical were added in a volume that was empirically calibrated to ensure complete absorption; adjustments were made to accommodate for solubility of chemicals as needed (Table 2-1). The concentrations listed in this report are the final concentrations within the media; for control vials, solvent alone was added. To avoid over-crowding of the populations while ensuring sufficient numbers for statistical purposes, at least three vials were established for each mutant at each concentration and the numbers were pooled for analysis. Due to the global toxic effects that occur in progeny of all genotypes at higher drug

concentrations, additional vials were established to obtain numbers that were sufficient for statistical analysis. Progeny were scored daily using markers carried on a balancer chromosome as either hemizygous mutant, as in the case of *Dwee1*, homozygous mutant, as in the case of *grp* and *mei-41*, or heterozygous wild-type, which carry the dominantly marked balancer chromosome, until all viable progeny had eclosed. Balancer chromosome homozygotes are non-conditional lethal. All crosses described in this report were raised at 25°C.

Statistics: The hemizygous or homozygous mutant progeny were calculated as a proportion the total population of mutant plus balanced heterozygotes, by counting the number of adult flies of each genotype. In the case of *mei-41*, which is an X-linked gene, only females were counted and mutant homozygotes were calculated as a proportion of the total female population. Sensitivity was assessed by comparing treated and control vials using Chi-squared analysis; for the purpose of discussion, $p < 0.01$ was considered significant, with the null hypothesis in each case being that the checkpoint mutant was not differentially sensitive to each drug we tested.

RESULTS

Preliminary experiments first established a range within which each of the drugs we tested is (or is not) effective in preferentially killing checkpoint-defective *Dwee1*, *grp* or *mei-41* mutant larvae (data not shown). For these preliminary tests, single vial crosses were treated with drug concentrations covering a 100-fold range, using previously published information to establish an initial concentration range whenever possible. These pilot experiments also empirically established an upper range of drug concentration that would be toxic for heterozygous larvae. The alleles that we used are all reported to be amorphic, therefore the mutants should completely lack the zygotic

function of each gene. Full names and general information for each of the chemicals used is listed in Table 2-1.

Hydroxyurea (HU) is an inhibitor of ribonucleotide reductase, an enzyme required for the synthesis of deoxyribonucleotides. At concentrations that impede but do not completely block DNA synthesis, stalled replication forks are thought to elicit a response similar to that generated by DNA damage, namely the induction of the ATR and ATM-dependent checkpoint pathways in humans. Previous investigations of *Dwee1*, *grp* and *mei-41* mutants indicates that the zygotic function of each of these genes is required for viability when larvae are exposed to HU at concentrations that wild-type larvae can tolerate reasonably well (Banga *et al.* 1986; Fogarty *et al.* 1994; Sibon *et al.* 1997; Sibon *et al.* 1999; Price *et al.* 2000). These observations have been interpreted as an indication of the essential role for these genes in the DNA replication or S/M checkpoint. We have extended these observations by making direct comparisons between the sensitivities of these three mutants to hydroxyurea. Surprisingly, we find pronounced differences in the sensitivities of the mutants to HU, with *grp-1* mutants appearing to be most sensitive, *Dwee1* mutants being intermediate and *mei-41*^{D3} mutants being least sensitive (Figure 2-1; Table 2-2) when tested at concentrations ranging from 2mM to 10mM.

Methyl methane sulfonate (MMS) is potent mutagen that acts by base methylation, causing transition mutations. *Mei-41* mutants have previously been reported to be sensitive to MMS (Boyd *et al.* 1976). Since Dwee1 and grp function in the signalling cascade that is initiated by mei-41, we expected that mutants for these genes would also be sensitive to this chemical. We have confirmed the observation that *mei-41* mutants are extremely sensitive to MMS, with significant differential lethality observed at concentrations as low as 0.05 mM (Figure 2-2C, Table 2-3). Homozygous *grp* mutants are also sensitive relative to heterozygous siblings but at higher concentration levels (above 0.5 mM MMS), as shown in Figure 2-2B. Much to our surprise, however, our

results indicate that the *Dwee1* mutants are not sensitive to MMS at concentrations ranging from 0.5mM to 1.5mM, levels at which general toxicity effects severely reduce the total numbers of progeny of all genotypes (Figure 2-2A, Table 2-3).

Cytosine β-D-arabinoside, commonly called ara-C, is a nucleoside analog used as an anti-cancer agent that can inhibit DNA repair either by direct effects on DNA polymerases alpha and delta or by incorporation into DNA (Chung *et al.* 1991). Human cells with compromised DNA damage checkpoints such as those found in tumors, are preferentially sensitive to the toxicity of this drug. All three checkpoint mutants tested were significantly sensitive to ara-C; *grp* mutants appeared to be most sensitive. At a concentration of 60mM, all *grp* homozygous mutants die before reaching adulthood, while a lethal dose for *Dwee1* mutant hemizygotes appears to be between 75mM and 100mM. The proportion of *mei-41* mutant homozygotes drops drastically between 15mM and 75mM, then appears to plateau until complete lethality is observed at 200mM (Figure 2-3; Table 2-4).

Cisplatin is an anti-cancer agent that functions by crosslinking adjacent guanine bases, causing a bend in the DNA helix towards the major groove. This distortion of the helix cannot be repaired in checkpoint-compromised cells, leading to selective destruction of tumor cells by an apoptotic process (Rice *et al.* 1988; Bruhn *et al.* 1992). All three Drosophila checkpoint mutants that we tested also showed significant sensitivity to cisplatin. Homozygous *mei-41* mutants exhibit significant lethality at concentrations as low as 0.06mM, whereas *Dwee1* and *grp* mutants are similarly sensitive to cisplatin with significant zygotic lethality observable at 0.4mM and 0.3mM concentrations, respectively (Figure 2-4; Table 2-5).

Methyl viologen, commonly called paraquat, is metabolized to generate reactive oxygen species (ROS) that can cause widespread cellular damage, including DNA damage. Given that the checkpoint mutants we are studying appear to be sensitive to

certain forms of DNA damage, it seemed reasonable to investigate whether they would also be sensitive to paraquat. We tested concentrations of paraquat that are lethal to mutants which are defective for the cytoplasmic superoxide dismutase gene (*cSOD*), a component of the oxygen radical defence system (Phillips *et al.* 1989). Unlike *cSOD* mutants, which are completely inviable when grown at concentrations ranging from 5mM to 15mM paraquat, *Dwee1* and *grp* mutants showed no apparent sensitivity to this drug (Figure 2-5; Table 2-6). *Mei-41* mutants did show moderate but statistically significant sensitivity at concentrations as low as 12.5mM, however. This sensitivity seems to plateau at about 15mM with only a 10% reduction in the proportion of homozygotes.

DISCUSSION

The ATR/Chk1 and ATM/Chk2 pathways were originally thought to be discrete kinase signaling pathways that are activated in response to distinct DNA damage signals or to disruption of DNA replication. Collectively they are now thought of as DNA structure checkpoints (Abraham 2001). A common mechanism of action by which these checkpoint pathways acts converges on a reciprocal inactivation of Cdc25 phosphatases and activation of Wee1-like kinases (Wee1 and Myt1 in metazoans). The consequence of this event is that phospho-inhibited Cdk1 cannot be activated by Cdc25, thereby blocking entry into mitosis, since mitosis is absolutely dependent on Cdk1 activity. This relatively simple linear view of the checkpoint pathways has been complicated over the past several years by accumulating evidence for functional redundancies and interactions between the ATR/Chk1 and ATM/Chk2 pathways as well as their involvement in other responses, such as apoptosis and DNA repair (Abraham 2001). Moreover, it has been recognized that functionally distinct signaling pathways, notably the stress-activated p38 MAP kinases, may also be able to independently regulate G2/M progression by modifying the same targets to block Cdk1 activation.

Data reported here suggest even further complexity in the cellular responses to DNA structure problems exhibited by multi-cellular organisms. The Drosophila checkpoint mutants we have used in our study are all thought to encode components of the canonical ATR/Chk1/Wee1 kinase pathway and all are thought to represent non-functional allelic variants. Since each of these genes encodes an essential component of the signaling pathway, a naive expectation was that they would behave similarly to various chemical compounds that damage DNA or interfere with DNA replication. This is not, in fact, what we have seen. Certain compounds (HU and MMS most strikingly, ara-C and cisplatin less so) elicit dramatic differences in the survival of mutants versus heterozygous siblings, suggesting that complex variables affect the viability of each of the checkpoint mutant genotypes we have examined.

The HU survival data described in this study have confirmed previous reports that each of the checkpoint mutant stocks we were using is sensitive to HU and demonstrated that our method for testing these drugs is valid and capable of generating statistically significant comparisons. There were pronounced differences in the sensitivities of each of the mutants to HU, with *grp/Chk1* mutants being most sensitive and *mei-41/ATR* being least sensitive.

MMS creates DNA damage via alkylation and is normally repaired by the excision repair pathways (Blank *et al.* 1994; Sobol and Wilson 2001; Tercero and Diffley 2001). Previous studies had established that *mei-41* mutants are sensitive to MMS exposure (Boyd and Setlow 1976; Mason *et al.* 1981) and we report here that grp/Chk1 mutants are also quite sensitive to this chemical. Although both genotypes are markedly sensitive to MMS, the $mei\text{-}41^{D3}$ mutant flies are roughly ten-fold more sensitive to MMS compared to *grp-1*. Surprisingly, especially in light of these results, $Dwee^{ES1}$ hemizygous mutants are not differentially sensitive to MMS at the highest concentration levels tested. These data suggest that *grp* and *mei-41* each play some unique role in the response to

DNA damage elicited by MMS, whereas *Dwee1* does not have an essential role in this process. Possible unique roles for *grp* and *mei-41* include modulating DNA repair or apoptotic functions.

In contrast to the results obtained with MMS exposure, our data also show that *Dwee1* function is required for viability in response to two other chemicals that are commonly used for chemotherapy and whose mechanism of action is also thought to involve DNA damage (ara-C and cisplatin). In fact, all three checkpoint mutants are sensitive to ara-C and cisplatin. *Dwee1* and *grp* mutants exhibit fairly similar degrees of sensitivity to ara-C, with the proportion of hemizygous or homozygous mutants steadily declining at concentrations of 100mM and 60mM ara-C, respectively. Mutants for *mei-41* exhibit a dramatic drop in viability at lower concentrations of ara-C, but complete lethality is not observed until concentrations reach between 150mM and 200mM. Ara-C is a therapeutic nucleoside analogue whose incorporation into DNA disrupts further DNA synthesis, triggering p53 and DNA-PK/Ku activation and apoptosis and ara-C cytotoxicity is enhanced in human wild-type *p53* cells versus *p53* null cells due to its facilitation of apoptosis (Feng *et al.* 2000; Achanta *et al.* 2001). It will be interesting to see if the sensitivity of Drosophila checkpoint mutants to ara-C can similarly be suppressed in the absence of Drosophila *p53* function, as would be expected by functional analogy with results from the mammalian system. In contrast to the results with ara-C, *mei-41/ATR* mutants were significantly more sensitive to cisplatin than *Dwee1* or *grp/Chk1* mutants, although the differences were less pronounced than those seen with ara-C.

The differential sensitivity of checkpoint mutants to different drugs may partially result from involvement of another Drosophila Wee1-like kinase called Myt1, in the checkpoint response (Cornwell *et al.* 2002; Price *et al.* 2002). Myt1 kinase activity is regulated by c-Mos and MAP kinase signaling pathways in Xenopus (Fattaey and Booher

1997; Palmer *et al.* 1998), and although it does have distinct roles in the regulation of mitotic events, it is conceivably also a target effector for the ATM/Chk2 and ATR/Chk1 checkpoint pathways. In Drosophila, Myt1 functions in parallel to Wee1 in its ability to phosphorylate cdc2 (Cornwell *et al.* 2002), and in certain cell types or in responses to specific chemicals, Myt1 kinase may compensate for the absence of Wee1 kinase function, thereby accounting for some of our results. We must also acknowledge the likely contribution of Drosophila homologs for the ATM and Chk2 kinases, in a parallel checkpoint pathway that can also respond to different DNA structural defects. Mutant alleles for these genes have recently become available (Silva, unpublished results), therefore similar analyses of these mutants as well as analyses of double mutant combinations may help to elucidate the complexities in the DNA structure responses that we have here documented.

We also investigated whether Drosophila checkpoint mutants might be sensitive to increased levels of oxygen free radicals, since these are thought to cause DNA damage in addition to other types of cellular damage. Surprisingly, our results do not support the initial hypothesis that *Dwee1* or *grp/Chk1* mutants would be sensitive to the oxygen radical-generating compound paraquat and we found only minimal differential sensitivity for *mei-41/ATR* mutants to this drug. *Drosophila*, like all other eukaryotes, has an oxygen defense system that scavenges and neutralizes oxygen radicals. Part of this system involves the activity of the copper/zinc-containing superoxide dismutase enzyme (cSOD). Unexpectedly, Drosophila *cSOD* mutants are far more sensitive to paraquat than any of the checkpoint mutants described in this report (Phillips *et al.* 1989). Presumably this result reflects the fact that the checkpoint mutants each have a fully functional oxygen defense system that is protecting them by scavenging paraquat-generated oxygen radicals before they can cause DNA damage.

It is clear from our results that mutations affecting different components of a DNA structure checkpoint confer very different sensitivities to the drugs we have tested, a finding that may be relevant to the diversity of responses observed in human cancer patients to these drugs. Conceivably in the future it may be possible to tailor chemotherapeutic regimens to individual patients by first determining the functional status of checkpoint genes in their cancer cells and then choosing drugs that preferentially affect specific genotypes. For example, our results suggest that individuals with defective *Chk1* alleles but normal *Wee1* and *ATR* alleles might benefit more from HU chemotherapy than individuals carrying mutations in *Wee1* or *ATR*. Although there is no data as of yet showing a propensity for any human cancer to display mutations in *ATR*, *Chk1* or *Wee1*, there is certainly a reasonable possibility that some types of cancer cells will be more dependent on this checkpoint pathway than the types of cancer in which mutations in *ATM* and *Chk2* genes have commonly been found.

As we have shown, this is a relatively inexpensive and sensitive method for assaying the effects of a range of compounds on biosystems. Other potential applications for our approach include the expansion of proteomic databases by establishing links to drug response studies done in model organisms as well as assessing the properties of newly synthesized chemicals. Although is not yet clear the extent to which information derived from tests of Drosophila (or other model organism) checkpoint mutants will be relevant for understanding the complexities of drug responses in mammalian cells, it is certainly of great interest and potential importance to pursue these investigations in genetically tractable and relatively simple experimental systems.

Table 2-1. General information on the compounds used in this analysis.

Compound Name	CAS[a]	PURITY	MW	SOLVENT	VOLUME ADDED
Cis-platinum (II) Diammine Dichloride (cisplatin)	15663-27-1	9.2% to 9.5% Nitrogen	300.0	0.9% NaCl	500µL
Cytosine ß-D-Arabinoside Hydrochloride (Ara-C)	69-74-9	~99% by HPLC	279.7	dH2O	500µL
Hydroxyurea (HU)	127-07-1	min 98% TLC	76.05	dH2O	300µL
Methyl methanosulfonate (MMS)	66-27-3	~99% (GC)	110.1	dH2O	300µL
Methyl viologen (Paraquat)	1910-42-5	NLT 98% (TLC)	257.2	dH2O	500µL

a. Chemical Abstract Services registry number

Table 2-2. Statistical analysis of *Dwee1*, *grp* and *mei-41* mutant survival on HU.

	concentration	X^2	P value	N value
Dwee1	0mM	N/A	N/A	289
	2mM	0.73	3.94E-01	303
	4mM	0.58	4.45E-01	246
	6mM	2.32	1.27E-01	191
	8mM	25.33	4.41E-07	138
	10mM	9.77	1.77E-03	28
grp	0mM	N/A	N/A	287
	2mM	0.00	9.59E-01	223
	4mM	>30	6.86E-09	382
	6mM	>30	2.74E-11	271
	8mM	>30	1.94E-09	138
	10mM	9.82	1.72E-03	25
mei-41	0mM	N/A	N/A	210
	2mM	0.00	9.84E-01	134
	4mM	4.17	4.12E-02	240
	6mM	19.60	9.24E-06	211
	8mM	17.96	2.27E-05	91
	10mM	15.18	9.79E-05	28

Table 2-3. Statistical analysis of *Dwee1*, *grp* and *mei-41* mutant survival on MMS.

	concentration	X^2	P value	N value
Dwee1	0mM	N/A	N/A	213
	0.5mM	1.60	2.05E-01	148
	0.75mM	0.06	8.08E-01	124
	1.0mM	1.58	2.08E-01	18
	1.25mM	0.02	8.84E-01	15
	1.5mM	0.01	9.23E-01	6
grp	0mM	N/A	N/A	270
	0.1mM	0.03	8.53E-01	255
	0.25mM	1.35	2.45E-01	442
	0.5mM	21.36	3.93E-06	232
	0.75mM	14.41	1.47E-04	63
	1.0mM	1.74	1.87E-01	6
mei-41	0mM	N/A	N/A	185
	0.005mM	3.85	4.98E-02	242
	0.0075mM	1.04	3.08E-01	251
	0.01mM	1.81	1.78E-01	281
	0.05mM	>30	1.19E-08	128
	0.075mM	>30	9.90E-14	102

Table 2-4. Statistical analysis of *Dwee1*, *grp* and *mei-41* mutant survival on ara-C.

	concentration	X^2	P value	N value
Dwee1	0mM	N/A	N/A	329
	10mM	0.47	4.91E-01	290
	35mM	5.44	1.97E-02	373
	50mM	16.53	4.79E-05	191
	75mM	18.16	2.02E-05	81
	100mM	5.45	1.96E-02	13
grp	0mM	N/A	N/A	193
	15mM	1.77	1.83E-01	239
	30mM	15.63	7.71E-05	250
	45mM	>30	1.38E-07	144
	60mM	>30	2.68E-07	105
	75mM	24.37	6.21E-07	94
mei-41	0mM	N/A	N/A	222
	15mM	0.12	7.31E-01	146
	75mM	>30	4.54E-14	189
	100mM	>30	7.80E-14	145
	150mM	>30	9.00E-08	62
	200mM	14.73	1.24E-04	14

Table 2-5. Statistical analysis of *Dwee1*, *grp* and *mei-41* mutant survival on cisplatin.

	concentration	X^2	P value	N value
Dwee1	0mM	N/A	N/A	187
	0.3mM	0.63	4.26E-01	244
	0.4mM	3.59	5.80E-02	99
	0.5mM	5.77	1.63E-02	80
	0.6mM	8.63	3.30E-03	104
	0.7mM	3.54	6.01E-02	11
	0.8mM	14.50	1.41E-04	79
grp	0mM	N/A	N/A	242
	0.15mM	0.01	9.08E-01	174
	0.3mM	6.47	1.10E-02	257
	0.45mM	5.55	1.84E-02	199
	0.6mM	11.58	6.67E-04	78
	0.75mM	6.07	1.37E-02	25
mei-41	0mM	N/A	N/A	195
	0.03mM	0.32	5.69E-01	156
	0.06mM	9.81	1.74E-03	189
	0.08mM	14.79	1.21E-04	193
	0.1mM	>30	2.42E-07	184
	0.13mM	>30	2.17E-11	173

Table 2-6. Statistical analysis of *Dwee1*, *grp* and *mei-41* mutant survival on paraquat.

	concentration	X^2	P value	N value
Dwee1	0mM	N/A	N/A	289
	5mM	1.14	2.85E-01	250
	7.5mM	1.11	2.93E-01	218
	10mM	0.24	6.27E-01	159
	12.5mM	0.04	8.34E-01	138
	15mM	2.57	1.09E-01	88
grp	0mM	N/A	N/A	251
	5mM	0.39	5.34E-01	217
	7.5mM	0.01	9.43E-01	200
	10mM	0.36	5.50E-01	214
	12.5mM	0.30	5.83E-01	167
	15mM	0.00	9.74E-01	135
mei-41	0mM	N/A	N/A	157
	10mM	0.02	8.88E-01	149
	12.5mM	2.79	9.51E-02	130
	15mM	8.73	3.12E-03	115
	17.5mM	8.05	4.55E-03	167
	20mM	7.52	6.10E-03	139

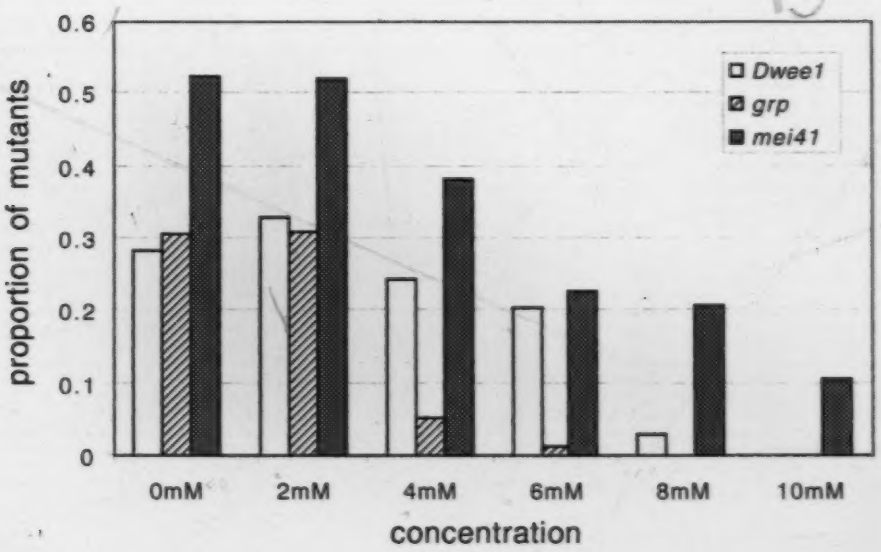

Figure 2-1. The proportion of *Dwee1*, *grp* and *mei-41* checkpoint mutants that survive to adulthood in relation to the total numbers of progeny (including their heterozygous siblings), when grown on the indicated concentrations of HU. Statistical analysis of the pooled data represented in this figure is found in Table 2.

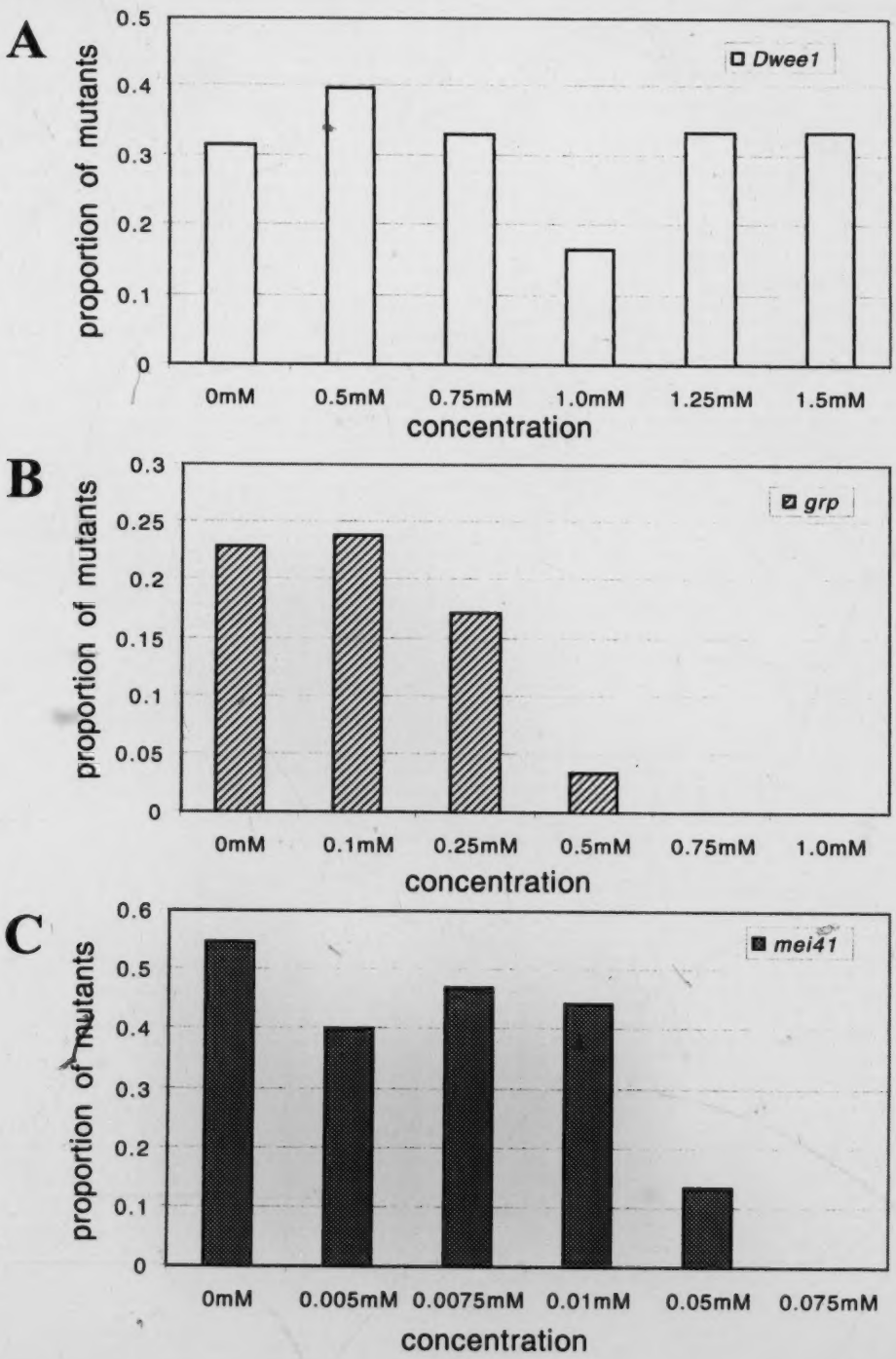

Figure 2-2. The proportion of *Dwee1*, *grp* and *mei-41* checkpoint mutants that survive to adulthood in relation to the total numbers of progeny (including their heterozygous siblings), when grown on the indicated concentrations of MMS. Statistical analysis of the pooled data represented in this figure is found in Table 3.

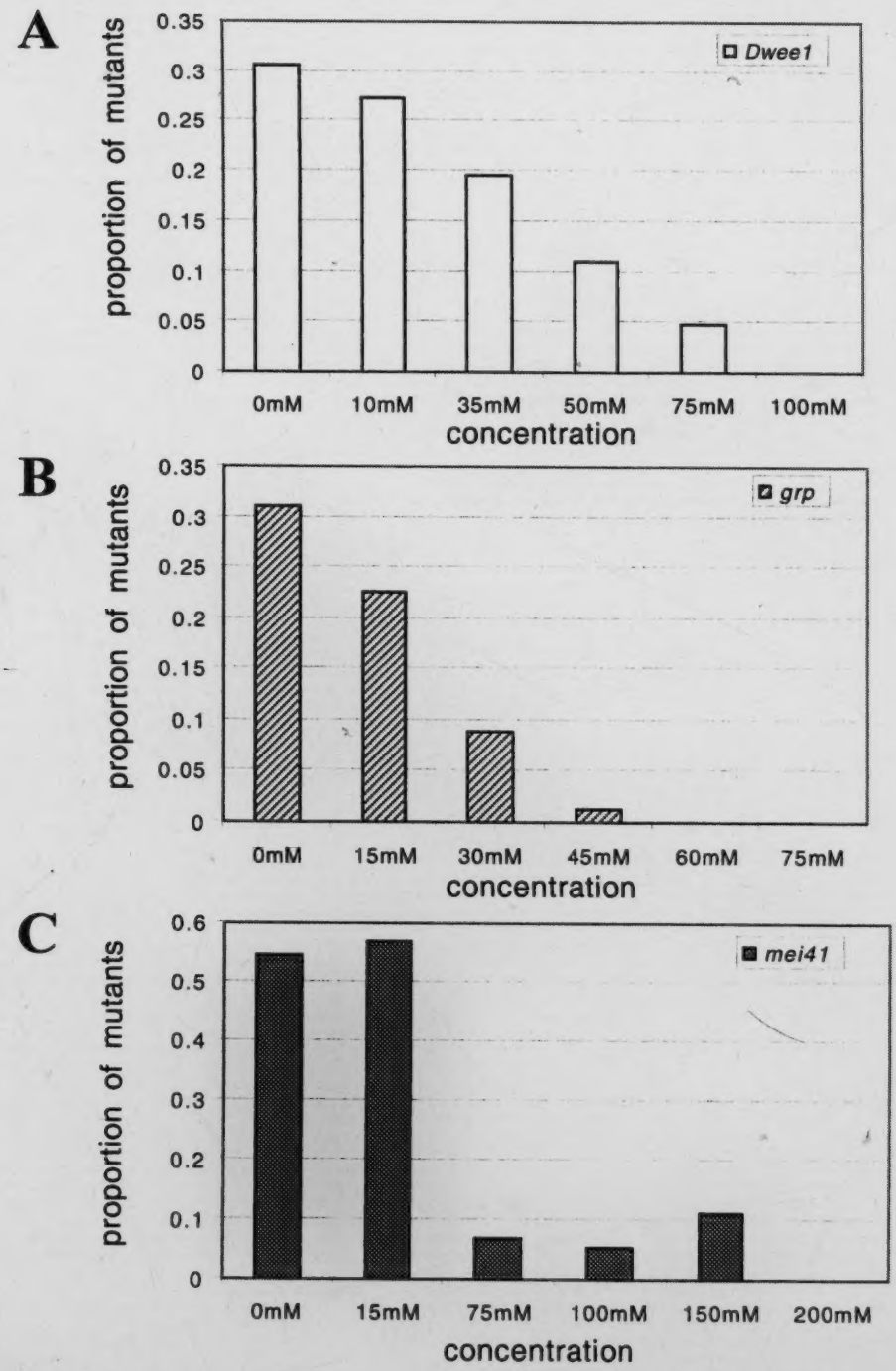

Figure 2-3. The proportion of *Dwee1*, *grp* and *mei-41* checkpoint mutants that survive to adulthood in relation to the total numbers of progeny (including their heterozygous siblings), when grown on the indicated concentrations of ara-C. Statistical analysis of the pooled data represented in this figure is found in Table 5.

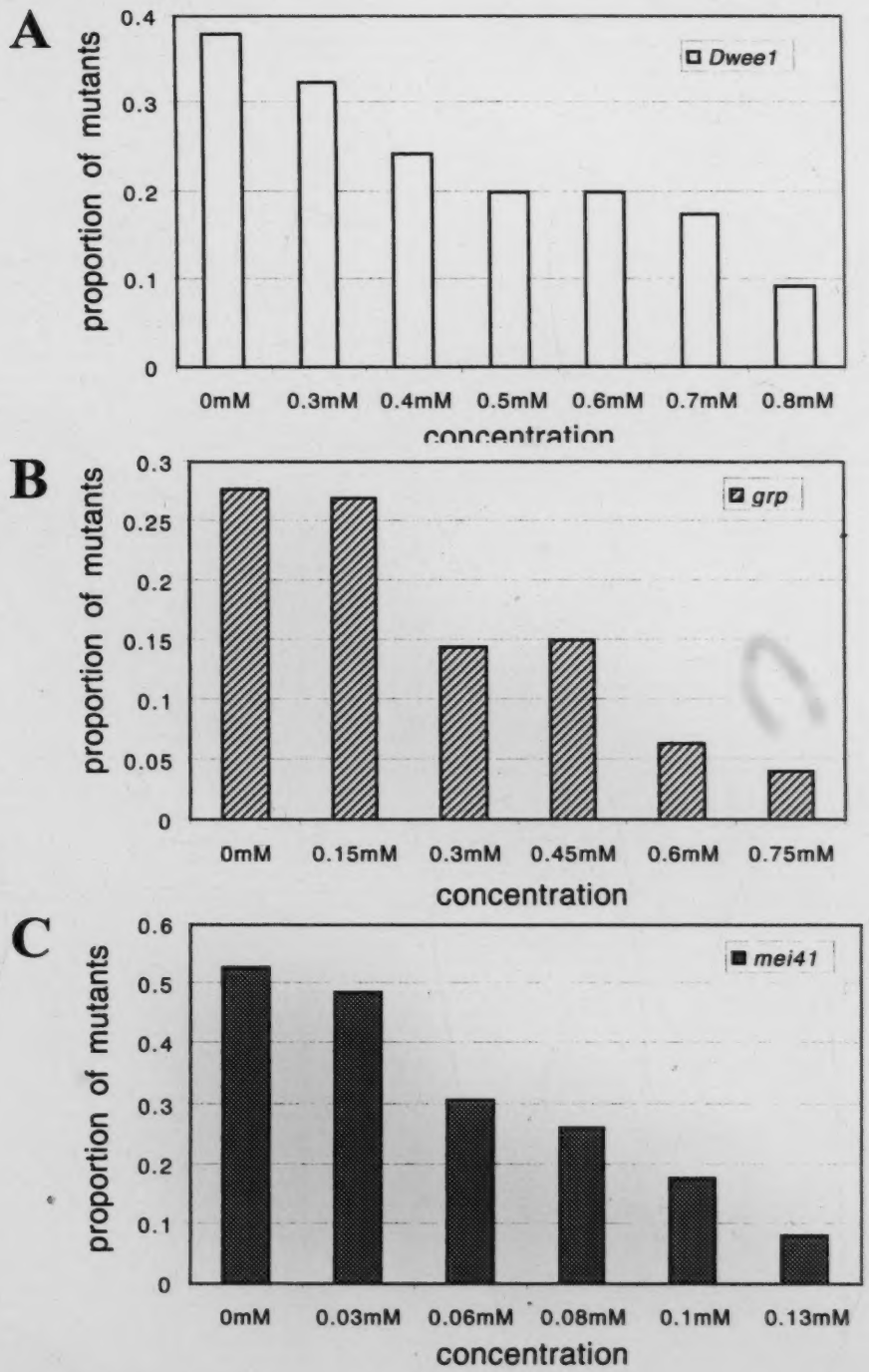

Figure 2-4. The proportion of *Dwee1*, *grp* and *mei-41* checkpoint mutants that survive to adulthood in relation to the total numbers of progeny (including their heterozygous siblings), when grown on the indicated concentrations of cisplatin. Statistical analysis of the pooled data represented in this figure is found in Table 6.

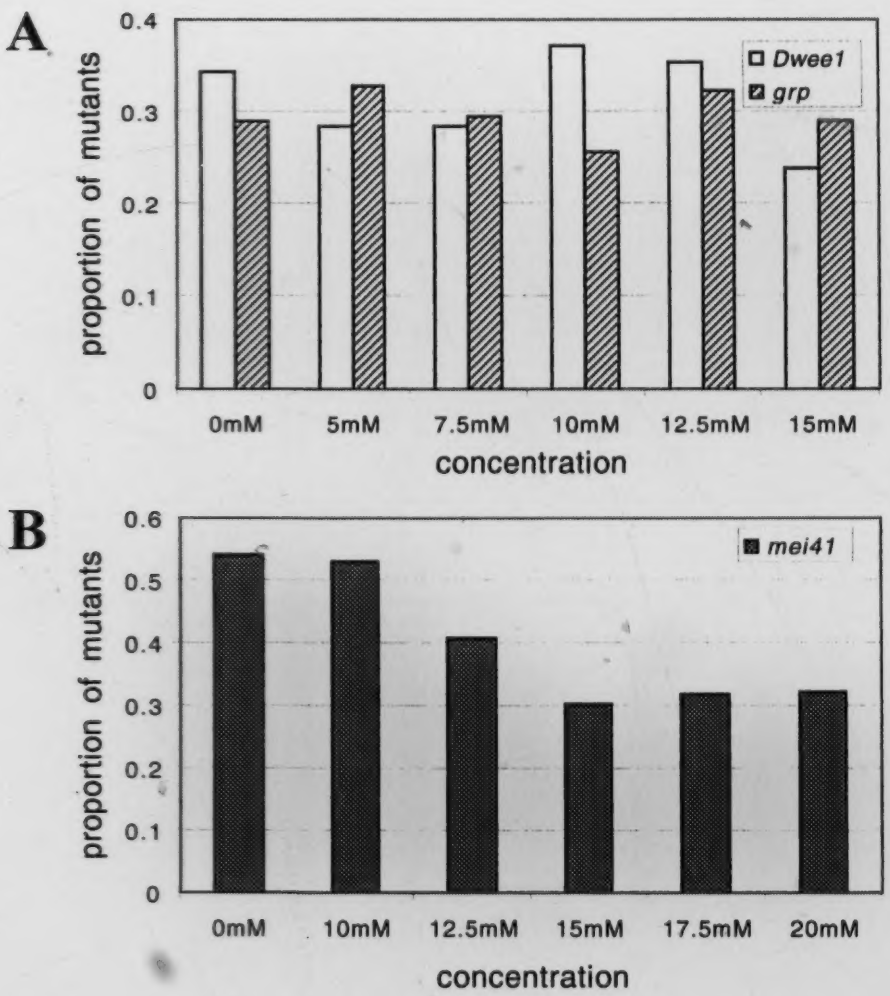

Figure 2-5. The proportion of *Dwee1*, *grp* and *mei-41* checkpoint mutants that survive to adulthood in relation to the total numbers of progeny (including their heterozygous siblings), when grown on the indicated concentrations of paraquat. Statistical analysis of the pooled data represented in this figure is found in Table 4.

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Chapter 3:

Mutants for Drosophila *ATM* (*dATM*) as a model for ataxia telangiectasia

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INTRODUCTION

Human ataxia telangiectasia (A-T) is a rare, recessive autosomal disorder associated with severe progressive degeneration affecting most major organ systems. Onset of symptoms usually occurs by two years of age, beginning with progressive degeneration of the central nervous system (CNS) manifested by dysfunction of motor coordination. Other symptoms include the occurrence of telangiectases (vaso-dilation) particularly around the eyes, immunodeficiency that leads to high rates of sinopulmonary infection, sterility, extreme sensitivity to ionizing radiation and high incidence of certain forms of cancer (Di Donato *et al.* 2001). Cellular phenotypes include DNA repair defects with consequent susceptibility to double strand breaks, cell-cycle checkpoint defects and chromosomal instability (Kastan and Lim 2000). Cloning and characterization of the gene that is defective in this disease, *ataxia telangiectasia mutated* (*ATM*), revealed that this gene is part of a large family of phosphotidalinositol-3 kinases, which include the S phase checkpoint genes *rad3* and *mec1* in *S. pombe* and *S. cerevisiae*, respectively (Savitsky *et al.* 1995; Uziel *et al.* 1996). ATM and its paralogue ATM- and Rad3-related (ATR) are thought to share the responsibilities as the apical protein kinases in the p53 and Chk1/Chk2 mediated mammalian cell cycle checkpoints (Shiloh 2001).

Much of what is known about the biochemical activities of ATM is derived from experiments in *ATM*-deficient cell lines. ATM itself is proposed to function in an

intricate array of signaling cascades that are designed to respond to DNA damage resulting from ionizing radiation at various points in the cell cycle by effecting either cell cycle arrest or apoptosis, depending on the circumstances (Kastan and Lim 2000; Abraham 2001). One of its roles is in the induction of the intra-S phase checkpoint; this pathway is distinct from the ATR/Chk1-mediated G2 checkpoint that is induced in response to incomplete replication and is triggered by hydroxyurea (Martinho *et al.* 1998; Zhao and Piwnica-Worms 2001). ATM triggers S-phase arrest through parallel but cooperative pathways, one of which involves a complex that includes the ATM substrate Nbs1 and the Nbs1 interacting protein, Mre11, while the other involves Chk2 and its substrates (Falck *et al.* 2002). Disruption of this checkpoint is the source of the ability of *ATM*-deficient cells to undergo radio-resistant DNA synthesis (RDS), the failure to arrest active DNA synthesis in the presence of DNA damage (Painter and Young 1980). Another mechanism of action for ATM is induced when DNA damage is incurred during G1 phase, resulting in p53 activation in an ATM- and Chk2-dependent manner (Banin *et al.* 1998; Canman *et al.* 1998; Matsuoka *et al.* 2000; Falck *et al.* 2001).

One characteristic that distinguishes the ATM/ATR kinases from other protein kinases is their susceptibility to functional inhibition by caffeine. It has been known for some time that a replication block triggered by hydroxyurea in yeast could be overcome by treatment with caffeine, but it was more recently confirmed that this checkpoint over-ride is achieved through inhibition of Rad3 (Moser *et al.* 2000) and vertebrate ATM and ATR (Blasina *et al.* 1999; Hall-Jackson *et al.* 1999; Sarkaria *et al.* 1999).

While biochemical experiments have provided a great deal of information regarding the function of ATM and its role in the manifestation of such a complex disease, it would be useful to study these roles *in vivo*. We have conducted a genetic screen to isolate mutations in the Drosophila homologue of *ATM* and here we describe the phenotypic characterization of eight alleles; these are the first *dATM* alleles to be

isolated. Mutants in *dATM* exhibit striking phenotypes that include defects in the peripheral nervous system, sensitivity to checkpoint perturbation through exposure to caffeine and decreased longevity relative to heterozygous siblings.

MATERIALS AND METHODS

Fly Strains and Genetics. The crossing scheme used to generate mutants for *dATM* is outlined in Figure 3-1. Mutations were induced in isogenized $p^p e^1$ males using 20-25mM ethyl methanosulfonate in a 1% sucrose solution according to Lewis and Bacher (1968) and screened over *Df(3R)PG4* described by Hing *et al.* (1999) on standard cornmeal and molasses medium containing 2mM caffeine. Mutants in the F2 generation were initially scored for lethality and female sterility on caffeine media, and then were re-tested to determine if lethality or sterility was dependent on caffeine. All stocks and crosses were maintained at 25°C. Other stocks used include *Df(3R)PB2*, *Df(3R)PA2*, $Hsc4^{XK1}$ and $Hsc4^{195}$ as described by Hing *et al.* (1999); $Hsc4^{Delta356}$, $Hsc4^{Delta16}$, $Hsc4^{Delta27}$, $Hsc4^{Delta27}$, $Hsc4^{Delta19}$ and $Hsc4^{Delta11}$ as described by Bronk *et al.* (2001); $Hsc4^{I-R35-R7}$, $Hsc4^{54.1}$ and $Hsc4^{II-R35-R7}$ as described by Mollaaghababa *et al.* (2001). Additional *Hsc4* alleles, $Hsc4^{l3929}$ and $Hsc4^{3550}$, as well as the $p^p e^1$ stock were obtained from the Bloomington Stock Center.

Electron and light microscopy. For environmental scanning electron microscopy, adult flies were fixed and desiccated using the critical point dry method according to Wolff (2000) then mounted directly and analyzed using a Philips XL30 ESEM ODP series scanning electron microscope. For transmission electron microscopy, bisected adult heads were fixed in 2% glutaraldehyde in PBS and post-fixed in 2% OsO_4 in PBS. Samples were desiccated in a graded ethanol series and then embedded in Spurr's resin with propylene oxide used as a transition solvent. Embedded heads were sectioned to approximately 60nm thickness with a Diatome diamond knife using a

Reichert-Jung ULTRACUT E ultramicrotome. Sections were collected in water on copper grids, stained with uranyl acetate and lead citrate, and viewed on a Philips Morgagni 268 transmitting electron microscope. Images were collected with a Soft Imaging System digital camera (model Megaview II). For analysis of the wing blades, whole wings were removed, mounted directly in Permount and pressed overnight. Samples were analyzed on a Zeiss Axioskop 2 microscope and images were collected using a Photometrics Sensys Optikon CCD camera.

RESULTS

A total of 19 lethal alleles and one recessive female sterile allele were generated that fail to complement *Df(3R)PG4* (Table 3-1). The *Df(3R)PG4* chromosome is deleted for a region of chromosome 3R that includes *dATM*; the distal breakpoint was mapped by molecular analysis to the 3' region of *Hsc4* located at 88E8 (Hing *et al.* 1999) and the proximal breakpoint maps cytologically to 88E1-2 (personal communication, G. Doughty). We conducted *inter se* complementation experiments to identify allelic mutations and also crossed each of the 20 mutant lines to two additional deficiencies that uncover parts of both *dATM* and *Hsc4* (Figure 3-2). Hemizygous and transheterozygous progeny from these crosses were then scored for their respective mutant phenotypes of lethality or sterility. Of these 20 alleles, 9 failed to complement $Hsc4^{Delta356}$ and $Hsc4^{Delta11}$. Eight of these nine alleles belong to a single complementation group, which includes seven lethal alleles as well as the single recessive female-sterile allele.

We next needed to distinguish between *dATM* mutants and *Hsc4* mutants since $Hsc4^{Delta356}$ and $Hsc4^{Delta11}$ uncover parts of both genes. *Hsc4* is a well-characterized Hsp70 cognate for which there are 24 documented mutant alleles (Hing *et al.* 1999; Bronk *et al.* 2001; Mollaaghababa *et al.* 2001). We obtained several of these and tested each of the nine alleles that failed to complement $Hsc4^{Delta356}$ and $Hsc4^{Delta11}$ against each

of the *Hsc4* mutant alleles. These alleles are listed in Table 3-2 along with the results of each cross and any molecular information that is available regarding the associated aberration. One of the nine alleles failed to complement all of the pre-existing *Hsc4* alleles tested; the remaining eight alleles, those that fall into the large complementation group described above, complemented all of the pre-existing *Hsc4* alleles that we predicted would not interfere with *dATM*. The 5' regions of these two genes are separated by a 530 bp intergenic space, making it likely that their transcriptional regulatory regions overlap, so we expected that there might be some small aberrations that affect expression of both genes. As such, when *dATM* alleles are put in transheterozygous combination with $Hsc4^{3550}$, a P-element insertion located in the first exon of *Hsc4* (Figure 3-2), approximately 10-20% of adults exhibit a visible phenotype that is described below and that corresponds to *dATM* mutant phenotypes. The remaining transheterozygotes display no apparent defects, indicating an incompletely penetrant effect. This P insertion has been documented as a strongly hypomorphic allele of *Hsc4* (Mollaaghababa *et al.* 2001) and we believe it also results in a very weakly hypomorphic allele of *dATM*. Collectively, these results suggest that we have isolated a single allele of *Hsc4*, which we designate as $Hsc4^{13}$, seven alleles of *dATM* that are hemizygous and transheterozygous lethal which we designate as $dATM^{1}$, $dATM^{2}$, $dATM^{3}$, $dATM^{4}$, $dATM^{5}$, $dATM^{6}$ and $dATM^{7}$, and one recessive sterile allele of *dATM*, which we designate $dATM^{stl}$.

$dATM^{stl}$ is a viable, female sterile allele of *dATM*: $dATM^{stl}$ was originally isolated as a caffeine-sensitive recessive female sterile allele, however subsequent tests determined that sterility does not depend on the presence of caffeine. Drosophila mutants for pre-mitotic checkpoint components such as *grp*, *Dwee1* and the *dATM* paralogue, *mei-41*, are maternal-effect lethal (Fogarty *et al.* 1994; Sibon *et al.* 1997; Price *et al.* 2000). The zygotic function of proteins encoded by each of these genes is not essential under normal

growth conditions, but early embryonic development requires a maternal contribution of mRNA that is deposited into the oocyte prior to fertilization. Consequently, females that are mutant for these checkpoint components are able to lay eggs, but embryos do not decelerate the rate of mitoses, a phenomenon that begins around cycle 11 in wild-type embyros, and mitotic catastrophe and death occur at cycle 13.

To determine if the female sterility observed in *dATM* mutants is due to the same effect, we collected eggs from fertilized, $dATM^{d1}$ hemizygous females. These females lay very few eggs, so large cages of mated females were established for collection. Gross examination of embryos collected from these cages revealed apparently normal development. This result was confirmed when embryos were left for an additional 3 days, allowing progeny to proceed through first and second larval instar at rates of development that were comparable to progeny derived from heterozygous females. From this data we concluded that the female-sterile phenotype originally observed in these mutants is not absolute, and that semi-sterility is likely due to a defect in meiosis and/or oogenesis, not the absence of a maternal contribution of ATM mRNA or protein to the embryo. Very few eggs are produced, but gametes that survive meiosis and oogenesis, if fertilized, proceed normally through embryonic development.

We also investigated whether hemizygous mutant males exhibit fertility defects. When wild-type virgin females were mated to hemizygous mutant males that had been cultured on 2mM caffeine, these crosses produce very few viable progeny when compared to controls using heterozygous males cultured under the same conditions. Hemizygous mutant males grown in the absence of caffeine appear to exhibit fertility levels that are comparable to controls. Thus we concluded that male zygotic *dATM* mutants also have a defect in meiosis and/or spermatogenesis that is dependent on exposure to caffeine.

Hemizygous *dATM*^{st1} mutant adults also exhibit visible phenotypes. The eyes appear rough, with few bristles, reduced numbers of ommatidia and fused ommatidia (Figure 3-3, compare A and B, C and D). We also see occasional "notching" of the wing-blade that is most often observed along the posterior margin (Figure 3-4A). These phenotypes are similar to those observed when *dATM* alleles are in transheterozygous combination with *Hsc4*^{P3550}, the P-insertion that affects both *dATM* and *Hsc4* and that is described above. In addition, hemizygotes cultured on media containing 2mM caffeine exhibit a slight reduction in viability. When cultured on caffeine, fewer *dATM*^{st1} hemizygotes survive to adulthood compared to both heterozygous siblings grown on caffeine and *dATM*^{st1} hemizygotes cultured under normal growth conditions.

Our final piece of data regarding *dATM*^{st1} mutants is more anecdotal than empirical, but is easily testable in future experiments. These mutants seem to exhibit decreased longevity; when crosses are initiated using *dATMst1* mutant hemizygotes, many of the parental hemizygotes are found dead within 10 to 20 days whereas heterozygotes survive for a minimum of an additional two to three weeks.

Lethal *dATM* alleles exhibit anti-morphic interactions with *dATM*^{st1}: The remaining seven alleles of *dATM* are unconditional lethal when in combination with *Df(3R)PG4*. Since *dATM*^{st1} is viable we expected that transheterozygous combinations of *dATM*^{st1} with these alleles would also be viable and would exhibit the visible defects that are observed in *dATM*^{st1} hemizygotes. Surprisingly, these combinations result in lethality and greatly reduced viability when compared with *dATM*^{st1} hemizygotes (Table 3-3). These data led us to conclude that the seven lethal *dATM* alleles are anti-morphic to varying degrees. *dATM*^{st1}/*dATM*^{3} and *dATM*^{st1}/*dATM*^{6} transheterozygotes, the two combinations that result in viable adults, exhibit phenotypes similar to, but more severe than, those seen in the *dATM*^{st1} hemizygotes. They have rough, reduced eyes and are female semi-sterile (Figure 3-3) and exhibit an enhanced wing phenotype compared to *dATM*^{st1} hemizygotes (Figure

3-4, compare A and B); they have not yet been assessed for male sterility defects. These flies also exhibit variable bristle phenotypes, in which some bristles are misoriented and thin, with a "Minute-like" phenotype (Figure 3-4, compare A and B). There is also a reduction in viability of these transheterozygotes that is enhanced when they are cultured on 2mM caffeine. When each of the seven lethal alleles is crossed to $Hsc^{Delta11}$, a deletion that removes the first exon of *dATM* (Figure 3-2), the hemizygotes are semi-lethal; there is greatly reduced viability among the hemizygotes. The few hemizygotes that do survive exhibit phenotypes similar to those seen in $dATM^{st1}/dATM^{3}$ and $dATM^{st1}/dATM^{6}$ transheterozygotes. Placement of each of the seven lethal alleles in transheterozygous combination with $Hsc^{Delta356}$, a deletion that removes the first eight exons of *dATM* and all of *Hsc4* (Figure 3-2), also results in semi-lethality in four of the seven combinations (Table 3-3). Again, the few that survive exhibit phenotypes that are indistinguishable from those seen in $dATM^{st1}/dATM^{3}$ and $dATM^{st1}/dATM^{6}$ transheterozygotes. In total, these genetic interaction data indicate that we have isolated a collection of *dATM* mutants with distinct phenotypic characteristics.

DISCUSSION

We have isolated eight EMS-induced alleles of *dATM* that in various combinations exhibit a range of phenotypes: lethality and semi-lethality that is enhanced when larvae are cultured in the presence of caffeine; disruption of components of the peripheral nervous system, including the eye and bristles; sterility that appears to be due to gametogenic and/or meiotic defects in both males and females; and disruption of wing blade development. Although we have not yet directly confirmed that these are indeed *dATM* alleles, our genetic data provides very strong evidence for this conclusion. Our alleles fail to complement $Hsc4^{Delta11}$ and $Hsc4^{Delta356}$, which affect only *dATM* and *Hsc4*, but do complement all of the *Hsc4* alleles tested, with the exception of $Hsc4^{P3550}$. We

propose that this insertion, which is just 550 bp upstream of the *dATM* start site, has a small effect on the expression of *dATM* in addition to its disruption of *Hsc4*. We have recently been generated a transgenic line carrying a complete genomic rescue construct for *dATM* that includes 3kb of upstream sequence, which should encompass the complete *dATM* regulatory region. We will use this transgenic stock to test for rescue of our mutant alleles. We are now sequencing each of the *dATM* alleles in order to identify the nature of the molecular lesions associated with each.

Studies of allelic variations of *ATM* in ataxia telangiectasia patients reveal that full manifestation of the disease results from complete absence of ATM function due to truncation of the protein (Concannon and Gatti 1997). *ATM* knock-out mice are also viable and exhibit many of the phenotypes that are characteristic of the human form of the disease, with the exception of the pronounced CNS defects (Barlow *et al.* 1996). Thus we were initially very surprised to find that mutations in *Drosophila ATM* could be lethal. However, further complementation analysis of these alleles revealed some results that conflict with this interpretation. Each of the seven lethal alleles appears to be antimorphic when put in transheterozygous combination with $dATM^{stl}$, since these transheterozygotes exhibit a more severe phenotype than $dATM^{stl}$ hemizygotes. However, when each of the same seven alleles are put in combination with $Hsc4^{Delta11}$, a deletion that removes the first exon of *dATM* as well as a region we expect includes the promotor, the mutant transheterozygotes are viable. Similar phenotypes are exhibited in $Hsc4^{Delta11}/Df(3R)PG4$ flies in which Hsc4 function is restored using a transgenic rescue construct (personal communication, W. Theurkauf). Intermediate results are seen when our *dATM* alleles are put in combination with $Hsc4^{Delta356}$, a deletion that removes the first eight exons of *dATM* and all of Hsc4; some transheterozygous combinations are lethal and some are viable (Figure 3-2, Table 3-3). These results lead us to one of two conclusions. Either $Hsc4^{Delta11}/Df(3R)PG4$ results in a null phenotype, or $Hsc4^{Delta11}$ has

some residual function and the null phenotype is lethal. We are in the process of developing and conducting of experiments to test each of these possibilities.

It is surprising that we have isolated so many anti-morphic alleles. This antagonism does not appear to be significant enough to affect heterozygotes cultured under normal growth conditions in any detrimental way, but does result in lethality of otherwise viable *dATM*^{tl} mutants. Biochemical studies of ATM have established its function within several large complexes including the BRCA1/ATM/SCM1 complex (BASC) (Wang *et al.* 2000), and more recently it has been suggested to function as a multimer, possibly a tetramer, within this complex (Smith *et al.* 1999; Scott *et al.* 2002). Various mutant forms of the protein have been generated by site-specific mutagenesis and *in vitro* testing has shown that kinase-dead versions, as well as missense mutations in regions distinct from the kinase domain, can lead to dominant interference of normal ATM kinase activity, as measured by phosphorylation of p53 (Scott *et al.* 2002). Given this information, it is not unreasonable to propose that translation of aberrant forms of *dATM* might also result in anti-morphic properties, so perhaps it is not remarkable that we have isolated so many alleles exhibiting this behavior.

In vivo studies suggest that ATM mediated-checkpoint responses are dose-sensitive. Haplo-insufficiency for murine *ATM* results in sensitivity to ionizing radiation and premature aging following exposure (Barlow *et al.* 1999). Although still controversial, some evidence indicates that carriers of human A-T are predisposed to develop certain forms of cancer, also suggesting dominant interference effects (Su and Swift 2000; Chenevix-Trench *et al.* 2002). Given these data, we intend to test our heterozygotes for sensitivity to ionizing radiation.

Many of the other phenotypes we observe in our *dATM* viable mutants lead us to propose that Drosophila could make an excellent model system for studying both ATM function and the manifestation of A-T *in vivo*. We have already established that there are

apparent defects in the development of the peripheral nervous system, as seen in the eye and bristles. We also have preliminary evidence for decreased longevity of these mutants. We plan to conduct assays for survival and temporal-dependant effects on locomotor function as indirect indicators of progressive neurodegeneration. We also plan to assess neurodegeneration in the CNS directly by making thick sections of the adult eye and brain over the course of their adult lives to look for progressive necrosis and apoptosis.

Mammalian ATM is known to directly interact with p53, Chk2, MDM2 and BRCA1, all of which play key roles in the cellular response to DNA damage (Khanna *et al.* 1998; Khosravi *et al.* 1999; Li *et al.* 2000; Lim *et al.* 2000; Matsuoka *et al.* 2000). Drosophila knockouts for *p53* (Rong *et al.* 2002) and mutant alleles of *Chk2* (Xu *et al.* 2001) have recently become available and we are eager to test these for interactions with our alleles of *dATM*. Both *p53* and *Chk2* null mutants exhibit no discernible phenotype in the absence of DNA damage or cell cycle perturbation but may be affected by a reduction in gene dose of *ATM*.

Studies of *ATM* knockouts in mice have been very informative, since they exhibit most of the hallmarks of A-T and provide an *in vivo* system for studying the function of ATM. For example, this system is useful in elucidating the mechanisms whereby disruption of *ATM* is capable of affecting such a diverse array of cellular processes. However, study of a genetically tractable system could also yield a wealth of information regarding the functions of ATM. For example, by conducting genetic screens for mutants that enhance or suppress the striking phenotype observed in the eye of *dATM* hemizygotes, it might be possible to identify novel players in various checkpoint pathways that both involve, and function in parallel to, ATM. The short life cycle and rapid development of Drosophila also make it feasible to use these mutants in high-throughput, *in vivo* analysis of drugs that could potentially be used in the treatment of A-

T. Despite apparent differences between the physiology of humans and flies, there is a high degree of conservation in the regulators of cell cycle progression, indicating that functional analysis of *dATM* will be both useful and insightful.

Table 3-1. Summary of the results of the EMS screen for mutants in *dATM*.

	sterility	lethality
Chromosomes screened	5700	6700
Mutants isolated	1	19
dATM alleles	1	7
Hsc4 alleles	n/a	1

Table 3-2. Mutant alleles of *Hsc4* that were tested for complementation with our EMS alleles.

Allele	Nature of aberration	complements *Hsc4*?	complements *dATM*?
$Hsc4^{l3929}$	P insertion in intron 1 of *Hsc4*	No	Yes
$Hsc4^{3550}$	P insertion in exon 1 of *Hsc4*	partially[a]	Yes
Df(3R)PB2	P insertion in exon 1 of *Hsc4*	No	Yes
Df(3R)PA2	deficiency that disrupts *Hsc4* function and deletes *dATM*	Yes	not tested
$Hsc4^{XK1}$	translocation or transposition that may affect *dATM*	Yes	Yes
$Hsc4^{195}$	gain of function EMS allele	No	not tested
$Hsc4^{Delta16}$	deletion of exon 1 and part of intron 1 of *Hsc4*	No	Yes
$Hsc4^{Delta27}$	deletion of part of P3550 and 250 bp distal	No	Yes
$Hsc4^{Delta19}$	deletion of a large region within P3550 insertion	No	Yes
$Hsc4^{54.1}$	antimorphic ENU allele	No	not tested
$Hsc4^{I\text{-}R35\text{-}R7}$	X-ray allele that is deleted for *Hsc4* but cytologically appears normal	No	not tested
$Hsc4^{II\text{-}R35\text{-}R7}$	X-ray allele; the nature of the associated aberration is unclear	No	Yes

a. 10-20% of transheterozygotes exhibited *dATM*-like visible phenotypes.

Table 3-3. Result of transheterozygous complementation testing of each of the *dATM* alleles with $dATM^{stl}$ and with three deficiencies that uncover part or all of $dATM^{+}$.

ATM allele	*Df(3R)PG4*	$Hsc4^{Delta11}$	$Hsc4^{Delta356}$	$dATM^{stl}$
$dATM^{1}$	0	I	0	0
$dATM^{2}$	0	I	0	0
$dATM^{3}$	0	I	0	I
$dATM^{4}$	0	I	I	0
$dATM^{5}$	0	I	0	0
$dATM^{6}$	0	I	I	I
$dATM^{7}$	0	I	I	0
$dATM^{stl}$	I	I	I	0[b]

a. Lethality is represented by 0, viability is represented by I.

b. Lethality of $dATM^{stl}/dATM^{stl}$ is likely due to a second-site mutation.

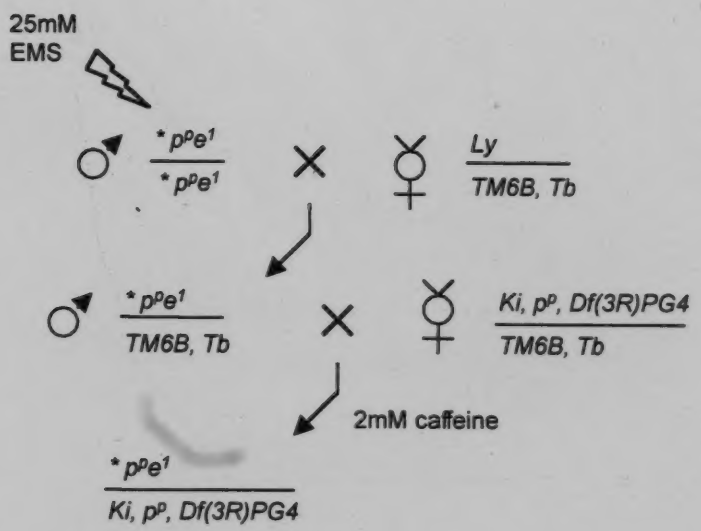

Figure 3-1. A genetic screen to identify mutants for *Drosophila ATM*. $p^p e^1$ males were mutagenized and crossed to balancer females, *en masse*. In the next generation, individual balanced males were collected and crossed to females carrying *Df(3R)PG4* and progeny were cultured on food containing 2mM caffeine. Hemizygous mutant progeny from this class were then scored for lethality and female sterility.

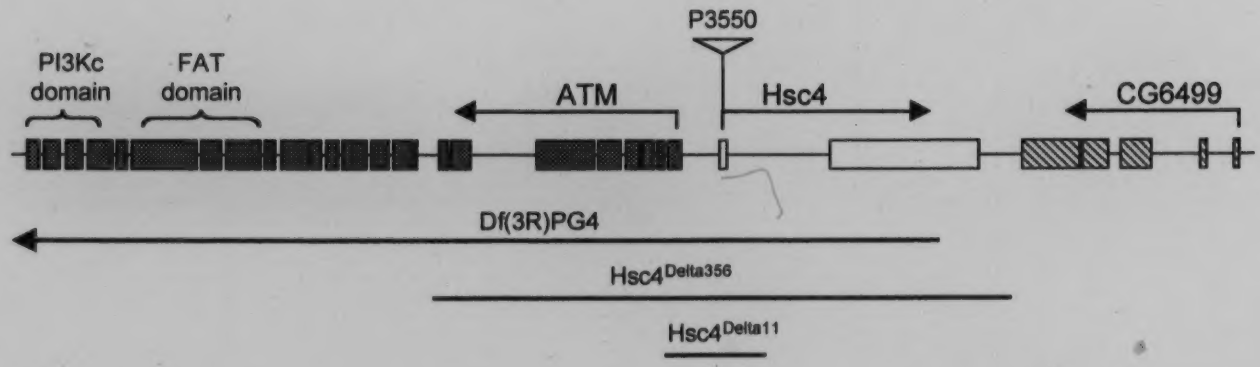

Figure 3-2. Genomic map of the *ATM* region. Arrows represent the direction of transcription for each gene, bars represent deficiencies and the open triangle represents the point of insertion of the P-element associated with $Hsc4^{3550}$ (P3550). The PI3Kc domain corresponds to the kinase domain, which has homology with the kinase domains of the canonical phosphoinositide 3-kinases, and the FAT domain corresponds to a region that is loosely conserved between ATM/ATR-like kinases and is named for **F**RAP, **A**TM AND **T**RRAP.

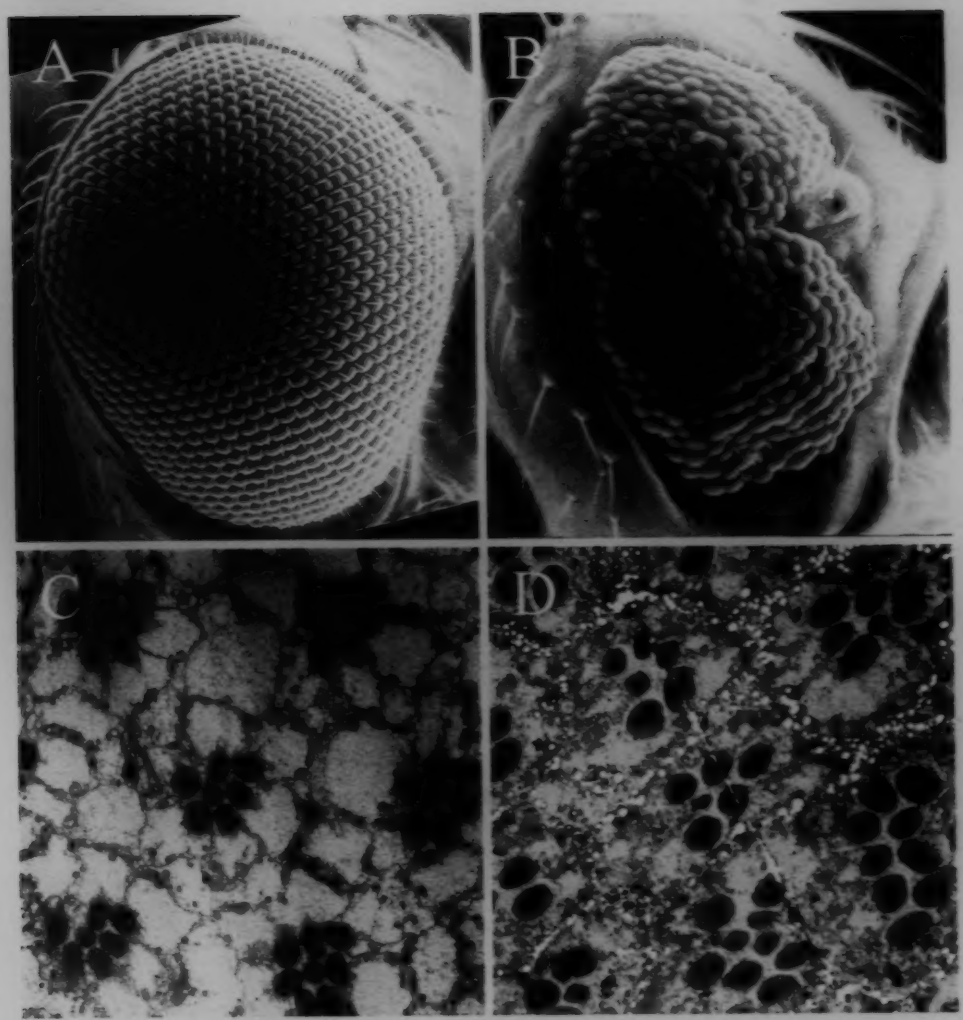

Figure 3-3. Environmental scanning and transmitting electron micrographs of *ATM*^{sterile}*/Df(3R)PG4* (B and D) and control (A and C) males. Hemizygous mutant adults have greatly reduced and rough eyes; ommatidia are often fused (compare A and B). Thin sections through the adult eye reveal mis-orientation of the ommatidia and fusion of individual rhabdomeres (compare C and D). Image 3-3A was kindly provided by D.M. Price.

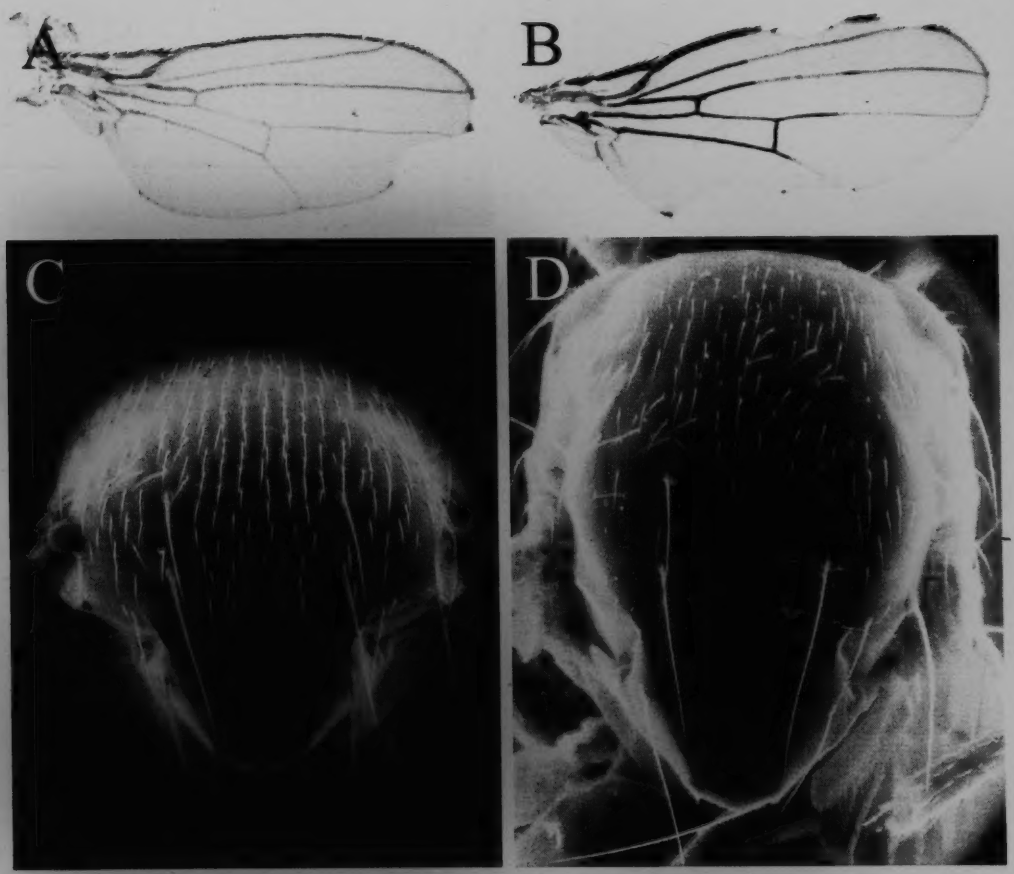

Figure 3-4. Environmental scanning electron micrographs and light micrographs of $ATM^{sterile}/Hsc4^{Delta356}$ (A), $ATM^{1}/Hsc4^{Delta356}$ (B and D) and wild-type (C) males. Hemizygous mutants exhibit variable phenotypes in which the wings are notched along just the anterior margin, or along both the anterior and posterior margins, of the blade; this phenotype is less severe in hemizygous $ATM^{sterile}$ mutants than hemizygous ATM^{1} mutants (compare A and B). Bristles in hemizygous ATM^{1} mutants are thin and brittle (compare C and D). Image 3-4C was kindly provided by D.M. Price.

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Chapter 4:

A novel genetic screen to detect Drosophila eye mutants that are sensitive to hydroxyurea and caffeine

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INTRODUCTION

Mitotic checkpoints were originally defined as controls that delay cell cycle progression in response to DNA damage (Weinert and Hartwell 1989). By that definition the genetic components of cell cycle checkpoints are not essential for viability under normal growth conditions. These controls have been extensively studied in *Schizosaccharomyces pombe*, where stimulation of the pre-mitotic checkpoint pathway by DNA damage or incomplete DNA replication results in inhibitory phosphorylation of Cdc2 (Cdk1), the mitotic cyclin-dependent kinase (Rhind and Russell 1998). It is now recognized that conserved genetic components of a similar pre-mitotic checkpoint also play an essential role during the early embryonic development of multicellular organisms (Fogarty *et al.* 1997; Sibon *et al.* 1997; Sibon *et al.* 1999; Brown and Baltimore 2000; Liu *et al.* 2000); these pre-mitotic checkpoints are not simply mechanisms for ensuring genome fidelity, but have evolved novel functions that are essential for coordinating cell proliferation with developmental events.

A method used successfully in yeast for isolating and studying checkpoint mutants is based on their failure to elicit appropriate repair and cell cycle arrest responses to DNA damage or to DNA replication blocks. The *rad* (radiation-sensitive) and *hus*

(hydroxyurea-sensitive) genes of *S. pombe* are examples of genes identified by this approach (Lehmann *et al.* 1991; Enoch *et al.* 1992; Carr 1995). A similar strategy was used to identify the *mus* (mutagen sensitivity) genes in Drosophila (Boyd and Setlow 1976). The majority of the *mus* genes appear to be components of DNA repair pathways, however *mus304* was recently characterized as a novel DNA damage checkpoint gene (Henderson 1999; Brodsky *et al.* 2000). Drosophila DNA replication checkpoint genes have also been identified based on their intrinsic requirement for some aspect of development. The *rad3* homologue, *mei-41*, was first identified as a gene required for normal meiotic recombination, whereas the *chk1* homologue, *grapes* (*grp*), was identified as having a maternal function that is essential for early embryonic development (Baker *et al.* 1976; Sullivan *et al.* 1993; Fogarty *et al.* 1994).

Since the response to DNA damage and DNA replication targets the same basic mitotic regulatory machinery that is required for all cell divisions, cell cycle checkpoint defects can result in non-conditional lethality or sterility, as the examples of *mei-41* and *grp* demonstrate. Thus, direct screens for mutagen-sensitive mutants will fail to recover null alleles of any checkpoint genes that are essential for viability. To circumvent this problem, we have developed a novel screening protocol that allows us to identify mutant alleles of essential genes that may also have cell cycle checkpoint functions. This protocol, adapted from a method developed by Stowers and Schwarz (1999), uses whole tissue clones of the eye to identify mutants that are sensitive to drug-induced developmental perturbation. The Drosophila eye is ideal for such a screen since it is not a tissue that is essential for the viability of the organism. The eye has specific, well-defined requirements for cell cycle regulation, which are evident in the progression of the morphogenetic furrow (MF). The MF is a dorsal-ventral indentation in the surface of the third instar eye imaginal disc that demarcates a transition from cells undergoing unpatterned divisions to the differentiation and organization of cells into precursors of the

adult ommatidia. Within the furrow cells become synchronized in G1 phase and a subset of these cells becomes organized into ommatidial pre-clusters (Wolff and Ready 1991a). Mutations in genes such as *roughex* and eye-specific alleles of *cdc25*^{stg} have implicated roles for both timed expression of cyclin-dependent kinase (Cdk1) inhibitors and activation of Cdk1 by dephosphorylation, respectively, in this patterning process (Tearle *et al.* 1994; Thomas *et al.* 1994; Foley *et al.* 1999; Mozer and Easwarachandran 1999). Cdc25^{stg} is responsible for driving cells ahead of the furrow through mitosis in preparation for G1 arrest, and Roughex is required for maintenance of G1 arrest within the furrow. As each new row of ommatidia emerges from the furrow, cells that are not already incorporated into pre-clusters undergo DNA replication followed by asynchronous cell division, in a process referred to as the "second mitotic wave" (SMW). These mitoses generate a pool of uncommitted precursor cells that are subsequently recruited to differentiate into the remainder of the 19 precisely arranged cells that ultimately form each adult ommatidium (Wolff and Ready 1991a).

In the hopes of identifying novel mutants displaying cell-cycle checkpoint defects, we screened for F1 flies with homozygous mutant eyes that conditionally exhibit a 'rough' phenotype when the larvae are raised in the presence of caffeine and hydroxyurea, two chemicals that are known to perturb the DNA replication checkpoint pathway. Hydroxyurea (HU) triggers the checkpoint by inhibition of ribonucleotide reductase, an enzyme required for the synthesis of deoxyribonucleotides. In *S. pombe*, HU delay of replication activates Rad3 and Cds1, two checkpoint kinases that promote the inhibitory phosphorylation of Cdk1 through stabilization of Mik1 (a Wee1-like kinase) and down-regulation of Cdc25 phosphatase activity (Lundgren *et al.* 1991; Rhind and Russell 1998; Moser and Russell 2000; Rhind and Russell 2001). Cells that are unable to deploy this checkpoint enter mitosis prematurely, causing cell death. Mutations in Drosophila homologues of these yeast checkpoint genes (*grp*, *mei-41* and *Dwee1*) are

also sensitive to HU exposure during development; concentrations of HU that do not affect heterozygous larvae are lethal to hemizygotes (Banga *et al.* 1986; Sibon *et al.* 1997; Sibon *et al.* 1999; Price *et al.* 2000). Caffeine, the other drug used in our study, has multiple pharmacological effects. Our interest is in its ability to inhibit Rad3 (Moser *et al.* 2000) as well as the vertebrate Rad3 homologues: ataxia telangiectasia mutated (ATM) and a related kinase, ATR (Blasina *et al.* 1999; Hall-Jackson *et al.* 1999; Sarkaria *et al.* 1999). We have observed that mutants for *Dwee1* are sensitive to caffeine with respect to whole organism viability (unpublished observations). In addition, pre-mitotic checkpoint mutants in *S. pombe* are also sensitive to caffeine and exposure of these mutants to caffeine in addition to hydroxyurea enhances this sensitivity (Wang *et al.* 1999). We reasoned that caffeine inhibition of the ATM-like kinases in Drosophila might render mutants for other checkpoint components sensitive to developmental perturbations, which could be detected by our screening protocol. Here we describe the results of a screen for conditional eye mutants on three chromosome arms, in which we detected novel mutations in five distinct loci.

MATERIALS AND METHODS

EMS mutagenesis: Mutagenesis was performed according to (Lewis and Bacher 1968). Males were exposed overnight to 20-25mM ethylmethanosulfonate (EMS) in 10% sucrose, then allowed to recover for 48 hours. The mutagenized males were then allowed to mate with females for a total of 5 days before being discarded.

Fly stocks and crosses: The basic crossing scheme and the fly stocks used in these experiments were previously described by (Stowers and Schwarz 1999). *Dwee1^{ES1}-FRT* and *grp^{1}-FRT* recombinant stocks were generated by crossing *w; Dwee^{ES1}/CyO* or *w; grp^{1} /CyO* males to *w; P{w^{+mC}=piM}36F P{ry$^{+t7.2}$= neoFRT}40A* females. In the next generation, females of the genotype *w; grp^{1}/P{FRT}* or *w; Dwee^{ES1}/P{FRT}* were selected

and crossed to males of the genotype *yw; P{w^{+mC}=GMR-hid}G1 P{ry$^{+t7.2}$=neoFRT}40A, l(2)CL-L1/CyO; P{w^{+m*}=GAL4-ey.H}SS5 P{w^{+mC}=UAS-FLP1.D}JD2*, and progeny were grown on G418 at a concentration of 3-4 mg/mL. Male progeny with a single *w+* transgene that were capable of FRT-specific recombination were established as balanced stocks and then crossed to the original *Dwee^{ES1}* or *grp^{1}* stock to test the non-balancer progeny for sterility to confirm the genotype. *FRT-mei-41^{D3}* stocks were generated according to a similar method using FRT inserts specific for the X chromosome.

Deficiency mapping was performed using the 2L and 3R deficiency kit stocks from the Bloomington Drosophila Stock Center. Further mapping of *huc29D-E* was performed using the following deficiencies: *Df(2L)N22-3* and *Df(2L)N22-5* , described by Wustmann *et al.* (1989), as well as *Df(2L)TE196x1, Df(2L)TE128x11, Df(2L)TE196x8, Df(2L)TE196x14* and *Df(2L)TE128x36*, obtained from G. Reuther. Further mapping of *huc95D-E* was performed using *Df(3R)crb87-4*, described by Wustmann *et al.* (1989). Other mutant stocks obtained from the Bloomington Drosophila Stock Center are: *raw^{k01021}*, described by Kania *et al.* (1995), *Acer^{k07704}*, described by Spradling *et al.* (1999), *Sema-1a^{k13702}*, described by Yu *et al.* (1998), *lmg^{03424}*, described by Taylor *et al.* (1997), *crb^{2}*, described by Tepass and Knust (1990), *Apc2^{DeltaS}*, as described by McCartney *et al.* (1999) and *jar^{1}*, described by Castrillon *et al.* (1993). *huc95D-E* was also tested for complementation with *Tsc1^{29}*, described by Gao and Pan (2001).

To test for complementation, mutant or deficiency-bearing males were crossed to *huc* mutant females for 2-3 days before adults were transferred to a fresh vial and allowed to deposit eggs for a further 24 hours. Larval progeny in the second vial were fed caffeine and hydroxyurea as described below and progeny from both vials were scored for the presence of any of four phenotypes: drug-dependent lethality, drug-independent lethality, drug-dependent reduced viability and drug-dependent eye defects.

Caffeine and hydroxyurea treatments: Mated females were allowed to deposit eggs on standard molasses and cornmeal media over a 24 hour interval; embryos were then allowed to develop for a further 24 hours. A stock solution containing caffeine and hydroxyurea dissolved in water was then added to the food at final concentrations of 2mM and 3mM, respectively, by scoring the media deeply with a clean metal spatula and adding the solution in a volume that had been calibrated to ensure complete absorption. The concentrations of caffeine and hydroxyurea used in these experiments were calibrated empirically to efficiently kill homozygous *Dwee1* mutant larvae without unduly compromising the viability of heterozygotes.

Histology and electron microscopy: For environmental scanning electron microscopy, adult flies were fixed and desiccated using the critical point dry method described by Wolff (2000b), then mounted directly and analyzed using a Philips XL30 ESEM ODP series scanning electron microscope. For transmission electron microscopy, fly heads of the desired genotypes were fixed and dessicated as above, then were embedded in Spurr resin with propylene oxide used as a transition solvent. Embedded heads were sectioned to approximately 60nm thickness with a Diatome diamond knife using a Reichert-Jung ULTRACUT E ultramicrotome. Sections were collected in water on copper grids, stained with uranyl acetate and lead citrate, and viewed on a Philips Morgagni 268 transmitting electron microscope. Images were collected with a Soft Imaging System digital camera (model Megaview II). To analyze eye imaginal discs, third instar larvae were dissected and fixed as described by Wolff (2000a) using 5% formaldehyde freshly diluted in 1X PBS. BrdU labeling and ELAV antibody staining was done according to the protocols described by Wolff (2000a). The discs were then stained with Hoechst (diluted in PBT to 1 μg/ml) for 3-4 min, rinsed in PBT and mounted in 80% glycerol with 0.02% sodium azide. The discs were then analyzed using a Leica TCS SP2 multiphoton confocal microscope.

RESULTS

Screening for F1 eye mutants that are sensitive to hydroxyurea and caffeine: The method devised by Stowers and Schwarz (1999) utilizes the FLP/FRT system to generate homozygous clonal eyes in an otherwise heterozygous fly. FLP expression is under the control of the GAL4/UAS system, which in turn is regulated by the *eyeless* promoter (EGUF). Thus, recombination between homologous chromosomes is induced only in eye precursor cells. The presence of an eye promoter-driven cell death transgene, GMR-*hid*, ablates all but the genotype of interest and results in selective proliferation of homozygous non-GMR-*hid* cells in the eye. We used the stocks assembled by Stowers and Schwarz (1999) to test whether this method could be utilized to identify conditional eye mutants, allowing us to isolate mutant alleles that might otherwise result in lethality or sterility of a homozygous mutant organism.

Since pre-mitotic checkpoint mutants are known to be sensitive to caffeine and hydroxyurea, we reasoned that a screen for Drosophila mutants that are sensitive to both these drugs might identify mutations in previously unidentified checkpoint genes. For this purpose, we selected for mutants with eye phenotypes that are dependent on the presence of hydroxyurea (HU) and caffeine, using a strategy that is described in Figure 4-1. In the F1 generation, progeny develop on media containing HU and caffeine. F1 males of the correct genotype that exhibit a 'rough-eye' phenotype in at least one eye were then selected for testing on normal media (without drugs). In the next generation, male progeny of the correct genotype with normal eyes were then isolated for further characterization. Different arm-specific FRT stocks were used in screens for each of chromosomes 2L, 2R and 3R.

We screened for drug-dependent eye mutants on three of the five large chromosome arms (2L, 2R and 3R) and found a total of nine mutants with drug-

dependent eye phenotypes (Table 4-1). The mutation frequencies varied according to the chromosome arm screened, ranging from ~0.6/1000 chromosomes screened (2L), ~0.3/1000 (3R) to 0/7500 (2R). A high percentage of the F1 generation males had at least one rough eye (~35%, N=24,500), but the vast majority of these mutants were non-conditional and presented a similar phenotype when larvae were grown on media without the drugs. More than 99.9% of these F1 generation eye mutants either exhibited drug-independent eye phenotypes or failed to incorporate the relevant mutation into the germ-line and were thus discarded. We have named the drug-dependent mutants that we isolated by this approach as ***h***ydroxy***u***rea/caffeine-dependent (*huc*) mutants. Each *huc* allele was initially given a numerical designation pending further analysis.

***huc29D-E* is an essential gene:** Eight mutants that were isolated in a screen of chromosome arm 2L show similar phenotypes in the eye. In the absence of HU and caffeine they all exhibit normal-looking eyes, whereas in the presence of HU and caffeine the eyes appear rough. We performed *inter se* complementation analysis and deficiency mapping of these mutants by scoring adults of the appropriate genotypes for drug-dependent lethality, drug-independent lethality, drug-dependent reduced viability and drug-dependent eye phenotypes. These experiments resulted in resolution of the eight 2L mutants into five complementation groups, one of which contains four alleles, as described in Table 4-2. These four alleles each fail to complement two overlapping deficiencies, localizing this gene to region 29D1-30C9. Further mapping using Df(2L)N22-3 and Df(2L)TE196x1 narrowed the region by excluding 29E2-6 to 30D1-2. We now refer to the gene as *huc29D-E*; confirmed and putative transcripts that map to this region are listed in Table 4-3 (Adams *et al.* 2000). All transheterozygous and hemizygous mutant combinations of *huc29D-E* result in drug-independent pupal lethality, thus *huc29D-E* is an essential gene. Transheterozygous mutant larvae are developmentally delayed by approximately three to four days compared to their

heterozygous balanced siblings. We tested the *huc29D-E* mutants against stocks carrying known mutations in the region 29D1-E6. Mutants for *raw*, *Acer*, *lemming* and *Sema-1a* all complement the *huc29D-E* mutants, indicating that the gene must be one of the other 19 predicted genes that map to this cytological region (Adams *et al.* 2000). The phenotypes and cytological locations for the remaining single-allele 2L mutants listed in Table 4-2 are inferred from complementation tests with single deficiencies and should thus be considered tentative, as the observed lethality may correspond to secondary lethal mutations rather than mutations associated with the conditional eye phenotypes.

Figure 4-2 illustrates the drug-dependent rough eye phenotype that we observe in *huc29D-E FRT40A/GMR-hid2L FRT40A CL2L* flies. In the presence of caffeine and HU the eyes are rough with ommatidia appearing disorganized and occasionally fused (arrow, Figure 4-2B). The mutant eyes also frequently exhibit indentations in the surface of the eye, suggesting a loss or disruption of the underlying tissue (arrowhead, Figure 4-2B). Consistent with this idea, electron microscopy of sections through the adult eye reveal irregularly spaced ommatidia, often with abnormal numbers of photoreceptors and accessory cells (Figure 4-2, compare C and D).

We wanted to test whether the drug-dependent *huc29D-E* eye phenotype could be discerned during early stages of ommatidial differentiation. Third instar *huc29D-E FRT40A/GMR-hid2L FRT40A CL2L* larvae that had been grown in the presence of HU and caffeine were dissected for this purpose and compared with discs from larvae grown without the drugs. DNA staining and BrdU labeling of eye imaginal discs revealed apparently normal morphology in the presumptive eye tissue, suggesting that most of the observed adult eye defects arise later during subsequent pupal development (data not shown). We also exposed larvae of this genotype to HU and caffeine independently to see if each drug has an independent effect on the eye morphology of the *huc29D-E* mutants. A slightly stronger eye phenotype was observed when larvae develop in the

presence of 2mM caffeine compared with exposure to 3mM HU. Exposure to each drug alone did not produce phenotypes as severe as exposure to HU and caffeine together; both drugs apparently contribute to the observed defects.

huc95D-E gene function is conditionally required for viability: A single mutant was found in our screen of chromosome 3R. Mapping crosses against two overlapping deficiencies localized it to region 95D9-F15 (see Table 4-2), since genetic analysis of Df(3R)crb87-4 places the proximal breakpoint at 95D8-9. A deficiency overlapping this region that complements this allele is *Df(3R)slo3*. The proximal breakpoint of this deficiency was localized to 95E6-7 by polytene analysis by (Atkinson *et al.* 1991) and we mapped it genetically to include *jaguar*, a gene located at 95F1. These data narrow the interval where this gene maps to 95D8-9 - 95E6-7. Consequently, we refer to this gene as *huc95D-E*. All confirmed and putative transcripts that map to this region are listed in Table 4-4 (Adams *et al.* 2000). Hemizygous *huc95D-E* mutants exhibit drug-dependent lethality, indicating that *huc95D-E* is a non-essential gene under normal growth conditions. We tested *huc95D-E* for complementation with lethal alleles of *Tsc1* and *Apc2*, two genes that map to region 95D-E. Both mutations complemented *huc95D-E* however, implying that the gene is one of 22 other genes predicted for the region (Adams *et al.* 2000).

In the absence of drugs, *FRT82B huc95D-E/FRT82B GMR-hid3R CL3R* flies appear normal, but in the presence of HU and caffeine the eye is dramatically reduced in size and grossly disorganized, with distortion or duplication of the surrounding tissues often seen (Figure 4-3, compare A and B). Curiously, these heterozygotes are also sensitive to chemical treatment at the level of whole organism viability. Despite the fact that the flies are heterozygous for the mutation, these individuals comprise just 5-10% of the total adult population in the presence of HU and caffeine; in comparison this

genotype represents 20 to 30% of populations not exposed to the drugs. The significance of this observation is unclear.

We examined eye discs from third instar *FRT82B huc95D-E/FRT82B GMR-hid3R CL3R* larvae that were grown in the presence of HU and caffeine. DNA staining (data not shown) and antibody staining against the neuronal marker ELAV (Figure 4-3, C-F) revealed defects in pattern formation behind the morphogenetic furrow. Although differentiation of the ommatidial precursors still initiates, the regular pattern of the pre-clusters is disrupted. Mutant *huc95D-E* discs from larvae grown on HU and caffeine are extremely fragile and thus sensitive to the physical manipulations required for dissection, making it difficult to visualize these defects. Some of the disruption we observe at this stage may be attributable to this fragility. There is also a striking difference in overall disc size that can be observed when similarly aged eye imaginal discs are compared to heterozygous controls (Figure 4-3, compare C and D). Mutant discs are significantly smaller but there does not appear to be any reduction in the size of the individual cells, indicating there must be fewer cells per disc (Figure 4-3, compare E and F). To explore the possibility that cells in these discs are not proceeding through the requisite number of divisions, we examined the pattern of BrdU incorporation, which is used to mark cells that are progressing through S phase. Compared to heterozygous controls, fewer cells are labeled with BrdU in the presence of HU and caffeine, and the pattern of staining that is seen ahead and behind the morphogenetic furrow appears to be disrupted and more diffuse relative to the controls (Figure 4-4, compare A and B, C and D). To assess the relative contributions of each drug to the overall phenotype, *FRT82B huc95D-E/FRT82B GMR-hid3R CL3R* larvae were also grown on media containing either HU or caffeine, independently. In the presence of 3mM HU alone a slightly rough adult eye phenotype was observed, which did not appear to be substantially different from the untreated eye. However, a dramatic reduction in eye size and number of ommatidia was observed in the

presence of 2mM caffeine (Figure 4-3, compare A and B); this phenotype is comparable to the phenotype observed in individuals that are treated with both HU and caffeine together. We tested to see if caffeine alone was also responsible for the drug-dependant lethality we observe in the hemizygotes. When deficiency bearing males were crossed to *huc95D-E* females and progeny were exposed to 3mM HU alone, hemizygotes constituted 32.5% of the total adult population (N=237), which is not substantially different from the proportion observed in the absence of treatment (34.6%, N=341). In the presence of 2mM caffeine alone, no hemizygotes survived to adulthood (N=419). In fact, complete lethality was observed when progeny were exposed to caffeine concentrations as low as 0.5mM (N=277), indicating that these mutants are primarily sensitive to caffeine.

Previously identified DNA replication checkpoint mutants do not exhibit rough eye phenotypes in response to HU and caffeine: The *mei-41*, *grp* and *Dwee1* genes encode known components of the DNA replication checkpoint that have previously been demonstrated to be sensitive to HU exposure at the level of organism viability (Banga *et al.* 1986; Sibon *et al.* 1997; Sibon *et al.* 1999; Price *et al.* 2000). We anticipated therefore, that eye development would be defective in these mutants if they were exposed to HU as larvae. To test this idea, we generated the appropriate $Dwee^{ES1}$*-FRT*, grp^{1} *-FRT* and $mei\text{-}41^{D3}$*-FRT* recombinant chromosomes and tested them for drug-dependent eye defects using the EGUF system. Surprisingly, none of the mutants tested produced adults with mutant eye phenotypes when exposed to HU and caffeine under conditions identical to those that do produce such phenotypes in the *huc* mutants that we have described here.

DISCUSSION

We have described a novel genetic screening protocol for detecting Drosophila mutants whose eye development is sensitive to hydroxyurea and caffeine and have shown

that mutations in either essential or non-essential genes can be identified by this approach. Of the new mutants identified in the screen, we were able to cytologically localize four pupal-lethal alleles of one essential gene (*huc29D-E*) and one allele of a non-essential gene that becomes essential for both organism viability and normal eye development when the mutant larvae are grown in the presence of HU and caffeine (*huc95D-E*).

The mutations described in this report seem to cause degradation of the eye tissue in response to caffeine and hydroxyurea rather than failure to initiate normal development. Despite the fact that the majority of the cells should be homozygous mutant, the third larval instar eye discs appear relatively normal when compared to their adult phenotypes. In the case of *huc29D-E*, cell divisions appear to proceed normally as assessed by DNA staining and BrdU incorporation, even in larvae grown on media containing HU and caffeine. By eclosion however the ommatidial morphology is clearly defective, producing mutant adult eyes with a "rough" phenotype. This result was unexpected, since we anticipated that *huc* mutants would exhibit defects throughout eye development. The *huc29D-E* gene also apparently serves a non-conditional essential role in the development of other tissues, since hemizygous mutants grown on normal media die during the pupal stage. This could involve roles in promoting cell growth or proliferation, since the hemizygous mutant larvae are developmentally delayed relative to their heterozygous siblings.

Unlike the *huc29D-E* mutants, we do see patterning and cell cycle defects in third larval eye discs isolated from *huc95D-E* mutants grown under restrictive conditions. The mutant discs are significantly smaller than controls and although ommatidial precursor cells seem to initiate neuronal differentiation normally, the arrangement of the clusters is obviously disrupted. Cell size is not reduced, indicating that the reduction in disc size is due to the presence of fewer cells. Concomitant with these data is the observation that S

phase progression is disrupted. The mutants apparently undergo additional degradation of eye tissue during subsequent pupal development, since the adult eye is dramatically reduced in size relative to controls; much more so than differences we see in the corresponding third larval instar eye imaginal discs. Hemizygous *huc95D-E* mutants can develop normally on ordinary media but are not viable when grown on media containing even low concentrations of caffeine, indicating that the caffeine-sensitive function is essential in many tissues and may indeed reflect a novel cell cycle checkpoint function for the *huc95D-E* gene.

We have not yet definitively established that any of the genes we have identified in this study are novel cell cycle regulators, nor have we excluded that possibility. The premise of our screen was that genes involved in the DNA replication or DNA damage checkpoints would exhibit HU- and caffeine-dependent mutant eye phenotypes. Since caffeine is known to inhibit Rad3/ATR kinases such as Mei-41 and ATM, checkpoint mutants that are exposed to this drug should be impaired in their ability to respond normally to a partial replication block caused by ingestion of HU. We reasoned that eye precursor cells in known checkpoint mutants would inappropriately enter mitosis and die, producing a rough eye phenotype. However, we have shown that mutations affecting the *mei-41/grp/Dwee1* checkpoint do not in fact compromise eye development when mutant larvae are grown in the presence of HU and caffeine. This result contrasts with data showing that *mei-41* mutants exhibit eye defects in response to DNA damage caused by ionizing radiation, as assayed by radiation-insensitive mitotic progression (Brodsky *et al.* 2000). This discrepancy may reflect differences in how eye developmental defects are being assayed. Alternatively, a resolution to this apparent paradox may be that redundant checkpoint mechanisms are able to compensate for DNA replication defects during eye development. This argument is supported by the fact that functional redundancies have been well documented for the DNA damage/DNA replication checkpoint in *S. pombe*

(Feilotter *et al.* 1992; Boddy *et al.* 1998; Boddy and Russell 1999; Rhind and Russell 2001).

We have, however, demonstrated evidence for the involvement of *huc95D-E* in regulation of cell cycle progression. Exposure to caffeine results in disruption of progression through S phase, seen in BrdU labeled discs, and mutant discs exposed to caffeine are comprised of fewer cells, but the stage of the cell cycle that is directly affected and the nature of this defect are unclear.

One of the more attractive candidates for the molecular identity of *huc95D-E* is a homologue of p38, also called MPK2. While the primary pathway for regulating G2 delay after genotoxic stress is the caffeine-inhibitable ATM/ATR cascade, there is evidence from mammalian systems that the p38 kinases respond to UV damage by inhibiting mitotic progression. Many forms of p38 are activated rapidly in response to UV damage and among its substrates are Cdc25B (Bulavin *et al.* 2001) and p53 (Bulavin *et al.* 1999; Takekawa *et al.* 2000). When the ATM/ATR cascades are inhibited by caffeine, it is reasonable to expect that the p38-dependant pathway would compensate, taking over the developmental roles normally played by the ATM kinases. Attempts to identify single base-pair mismatches between annealed single strands of MPK2 coding sequence amplified from *huc95D-E* and wild-type genomic DNA failed to reveal a mutation. This does not exclude the possibility that *huc95D-E* is the Drosophila homologue for p38, since a mutation could exist in the regulatory region. We are currently exploring this speculation in our attempt to conclusively identify the molecular locus that corresponds to *huc95D-E*.

The mutations we have identified could also be affecting genes that are involved in the regulation of apoptosis. Pupal eye development is characterized by extensive programmed cell death, and drug-induced perturbations of this program could conceivably result in excessive or inappropriate cell death and degradation of tissue

(Wolff and Ready 1991b). One of the pharmacological effects of caffeine is to stimulate the release of internal calcium stores, (Szalai *et al.* 1999) and Chinese hamster ovary cells exposed to caffeine undergo apoptosis (Pan *et al.* 2000). The subsequent degradation of eye tissue seen in the caffeine-sensitive *huc95D-E* mutants could therefore be a consequence of failure to protect cells from caffeine-induced apoptosis. A related possibility is that the sensitivity to HU or caffeine observed in the *huc* mutants is the result of a defect in a membrane transport process that either prevents the influx or directs the efflux of metabolically disruptive molecules that can result in cell death.

The classical genetic screen remains the most powerful tool for the functional characterization of genes. The Drosophila genome project has provided a comprehensive description of genes predicted to be involved in various cellular processes (Adams *et al.* 2000); however, reliance on structural homology for the elucidation of gene function has significant limitations since direct comparisons between organisms can often be misleading. For example, roles for vertebrate Chk1 and Cds1 in the DNA damage and DNA replication checkpoints differ significantly from the functions observed in *S. pombe* (Guo and Dunphy 2000). RNA interference is increasingly popular as a method to assign gene function without the necessity of isolating mutations; however, many genes are not amenable to this strategy and those that are can be subject to substantial phenotypic variability (Bass 2000). Hence, the analysis of heritable mutations is still the method of choice, whenever possible. We have described a novel genetic screening method that in principle could be used to identify mutants that are sensitive to any chemical or other environmental stress. A significant drawback to this method is the fact that the majority of mutants isolated in the F1 generation have drug-independent eye defects, reflecting the large numbers of genes in the genome with roles in normal eye development. Despite this important caveat, we have demonstrated that our method is capable of identifying

mutations in genes that can respond to different types of genotoxic stress, alleles of which might otherwise be difficult or impossible to isolate.

Table 4-1. Results of mutagenesis of each chromosome arm.

Chromosome arm	2L	2R	3R
No. chromosomes screened	13 500	7500	3000
No. mutants isolated	8	0	1

Table 4-2. Putative cytological locations of the *huc* mutants and phenotypes identified in mutant hemizygotes.

Allele	Cytological location	Phenotype	Associated deficiencies
huc29D-E (2, 14, 18, 19)	29D01; 29E02-06	pupal lethal	Df(2L)N22-5
		(HU/caffeine independent)	Df(2L)N22-14
2L-11	unknown	N/A	N/A
2L-12	25D02-04; 24D03-04	lethal	Df(2L)ed1
2L-16	22F03-04; 23C03-05	reduced viability[a]	Df(2L)C144
2L-20	36E04-F01; 38A06-07	lethal	Df(2L)TW50
huc95D-E	95D08-09; 95E02-06	HU/caffeine dependent lethal	Df(3R)crb87-4,
			Df(3R)crb-F89-4

a. In the absence of HU and caffeine, hemizogotes constitute 62.2% of the total population (N=335); in the presence of HU and caffeine, hemizygotes constitute 30.7% of the total population (N=234).

Table 4-3. Putative transcripts that map to region 29D-E of the second chromosome.

Gene Name	Map Position	Putative Function	Notes
CG10595	29D1-D1	motor protein	myosin ATPase; also called *dachs*
CG13088	29D1-D1	unknown	homology to *D. melanogaster* CG13770 (e=7.1e-17); ligand binding or carrier protein
CG13094	29D1-D1	diuretic hormone	also called Dh31; involved in fluid secretion
CG13095	29D1-D1	endopeptidase	homology to *H. sapiens* pepsin A precursor (e=3.7e-82)
CG13096	29D1-D1	unknown	--
CG13097	29D1-D1	unknown	homology to *H. sapiens* nucleolar ribonucleoprotein MPP10 (e=8.2e-79)
CG13089	29D1-D1	unknown	homology to *S. cerevisiae cdc91* (e=2.1e-19)
CG13098	29D1-D1	ribosomal protein	also called mRpL51; mitochondrial protein
CG13090	29D1-D1	enzyme	contains a cell-cycle control phosphatase domain; homology to *H. sapiens* molybdopterin synthase sulphurylase (e=5.9e-98)
CG13091	29D1-D1	unknown	involved in oxidative stress response
Acer	29D1-D2	peptidase	complements *huc29D-E*
Img	29D2-D3	unknown	complements *huc29D-E*
CG17834	29D4-D4	unknown	--
Sema-1a	29D5-E2	cell adhesion	complements *huc29D-E*
Glt	29E2-E2	cell adhesion	homology to *H. sapiens* carboxylesterase (e=8.0e-33)
CG9287	29E2-E3	carboxylesterase	homology to Glt (e=1.8e-74)

Table 4-3 cont'd

Gene Name	Map Position	Putative Function	Notes
CG9289	29E3-E3	cell adhesion	contains a carboxylesterase; homology to Glt (e=7.2e-80)
CG9296	29E3-E3	cGMP-specific	homology to *M. musculans* rhodopsin sensitive phosphodiesterase cGMP phosphodiesterase (e=2.3e-48)
CG9298	29E3-E3	unknown	--
CG9314	29E4-E4	enzyme	homology to *H. sapiens* catalase (e=4.3e-159)
Hnf4	29E3-E4	steroid hormone receptor	homology to *M. musculans* hepatocyte nuclear factor (e=8.1e-97)
raw	29E5-E5	unknown	also called CG9321; complements *huc29D-E*
CG12438	29E6-E6	unknown	--

Table 4-4. Putative transcripts that map to region 95D-E of the third chromosome.

Gene name	Map position	Putative Function	Notes
CG10694	95D9-95D9	DNA repair protein	homology to *S. cerevisiae* rad23 (e=1.2e-21)
CG10716	95D9-95D10	translation factor	also called syntaxin1A; complements *huc95D-E*
CG18428	95D10-D10	unknown	--
CG13605	95D10-D10	DNA binding protein	contains putative zinc-finger domain
CG13607	95D10-D10	unknown	--
CG6129	95D11-E1	motor protein	homology to *H. sapiens* NEK2-assoc. protein (e=2.0e-73)
CG5463	95E1-E1	electron transfer	homology to *H. sapiens* Gln-tRNA (e=1.6e-102)
Tsc1	95E1-95E1	cell cycle regulator	complements *huc95D-E*
sec10	95E1-E1	motor protein	homology to *H. sapiens* brain secretory protein (e=5.7e-148); involved in vesicle trafficking
CG6164	95E1-E1	unknown	--
CG6173	95E1-E1	cell adhesion	homology to *H. sapiens* Kallmann syndrome protein precursor (e=4.9e-31)
Mpk2	95E1-E1	protein kinase	homology to *M. musculans* mitogen activated protein kinase 14 (e=5.2e-134)
CG6178	95E1-E1	enzyme	homology to *P. pennsylvanica* luciferase (e=1.0e-101); putative involvement in circadian regulation
CG5501	95E1-E2	actin binding	putative myosin protein
CG6182	95E2-E3	unknown	--
CG13608	95E3-E3	unknown	homology to *H. sapiens* MRP-S24 (e=3.4e-24)

Table 4-4 cont'd

Gene name	Map position	Putative Function	Notes
Apc2	95E3-95E3	tumor suppressor	complements *huc95D-E*
CG5510	95E3-E3	chaperone	homology to *H. sapiens* GP36B (e=1.7e-75)
CG13606	95E5-E5	unknown	--
CHORD	95E5-E5	ligand binding or carrier	putative CHORD containing protein (zinc binding)
CG5515	95E5-E5	protein kinase	homology to *H. sapiens* CGI-24 (e=4.9e-32)
CG5524	95E5-E5	DNA repair protein	homology to *S. pombe* rad18 (e=1.6e-71)
CG6204	95E5-E5	DNA binding	homology to *S. pombe* hypothetical ATP binding protein (e=6.0e-53)
CG5534	95E6-E6	enzyme	transcription factor
CG17786	95E6-E6	unknown	--

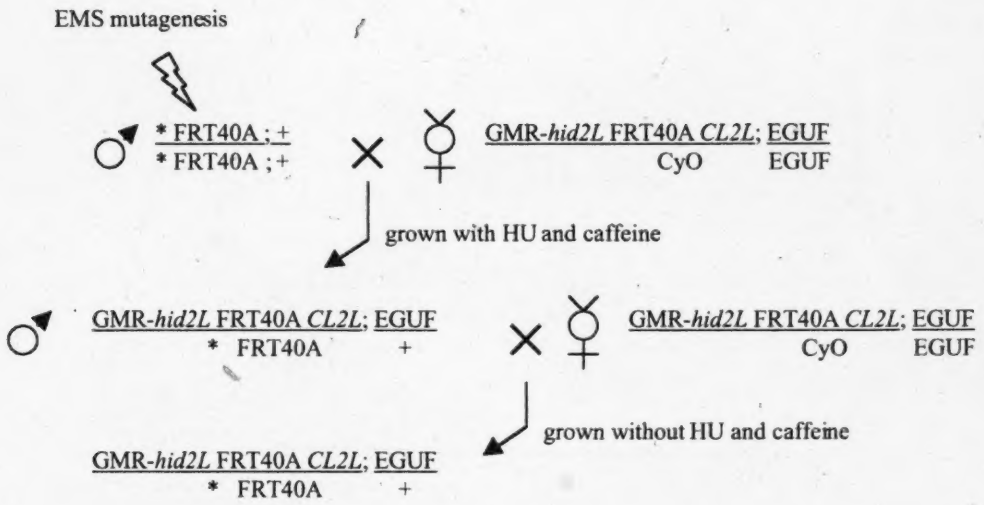

Figure 4-1. A genetic screen of chromosome 2L to identify mutants that are sensitive to hydroxyurea and caffeine during eye development. Eye-specific recombination between centromerically located FRT sites, in combination with eye-specific expression of the apoptotic inducing gene *hid*, results in eyes that are essentially a clone of a single genotype; the genotype of the remainder of the organism is heterozygous. F1 larvae are grown in the presence of HU and caffeine. Males exhibiting an eye phenotype are selected and crossed again to EGUF females and progeny are grown in the absence of HU and caffeine. Male progeny from this cross that exhibit a wild-type or near wild-type phenotype were isolated for further analysis. Similar methods were applied in screening chromosomes 2R and 3R, using arm-specific FRT stocks.

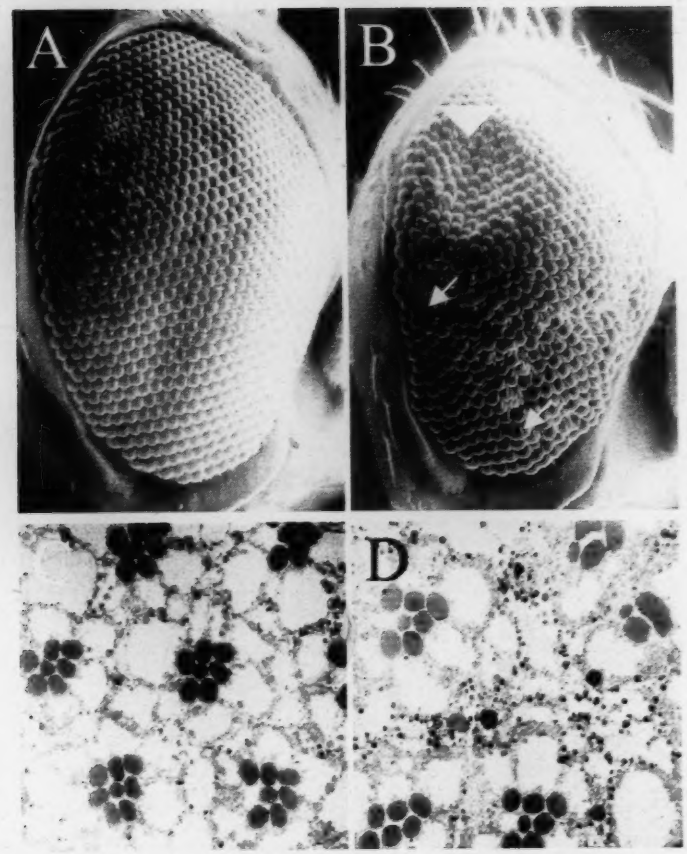

Figure 4-2. Scanning and transmission electron micrographs of eyes from *huc29D-E FRT40A / GMR-hid2L FRT40A CL2L; EGUF/+* flies grown in the absence (A and C) and presence (B and D) of HU and caffeine. When larvae are grown in the presence of HU and caffeine, adult eyes exhibit a 'rough' phenotype, ommatidia are fused (arrows) and the eyes are smaller. In approximately two thirds of eyes there are indentations as seen here (arrowhead in B). Sections through an adult eye reveal irregularly spaced ommatidia with variable numbers of photoreceptors and accessory cells (compare C with D).

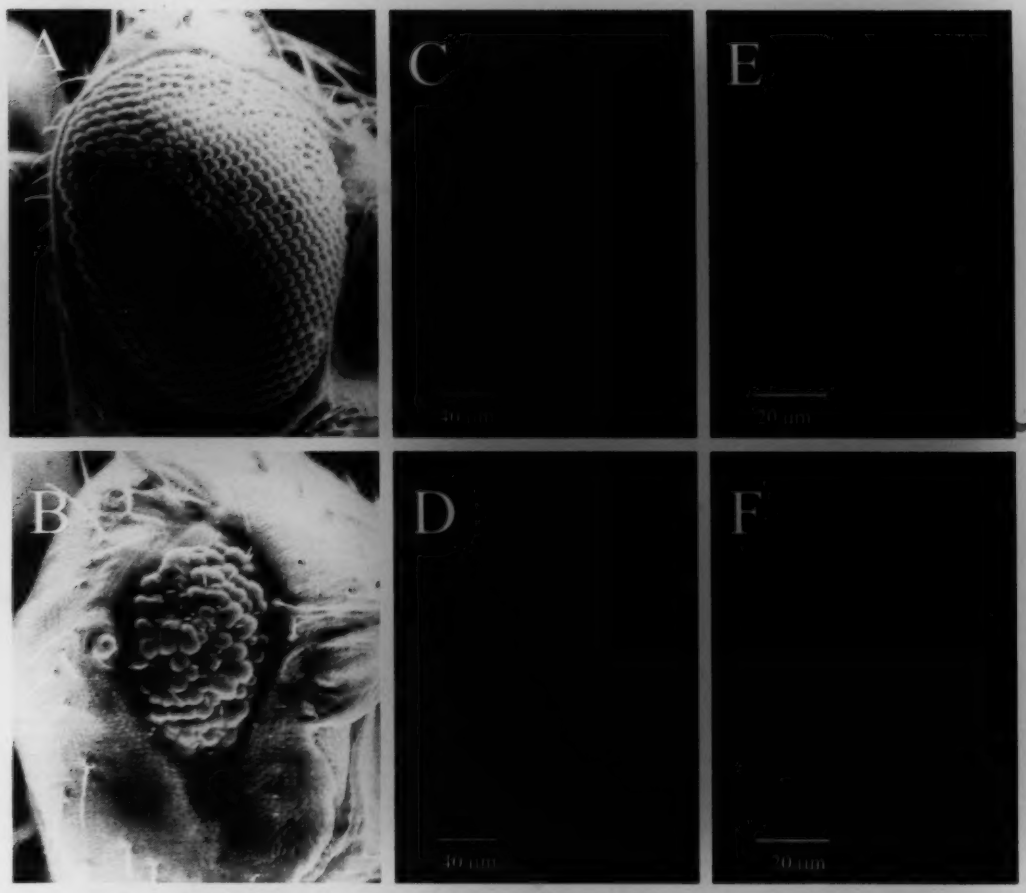

Figure 4-3. Scanning electron micrographs of adult eyes from individuals of the genotype *EGUF/+; FRT82B huc95E-D/ FRT82B GMR-hid3R CL3R* grown in the absence (A) and presence (B) of caffeine; the phenotype depicted here is indistinguishable from the phenotype that is observed when individuals are grown in the presence of both HU and caffeine. Eye imaginal discs from individuals of the genotypes *EGUF/+; FRT82B huc95E-D/ TM3 Ser* (C and E) and *EGUF/+; FRT82B huc95E-D/ FRT82B GMR-hid3R CL3R* (D and F) grown in the presence of caffeine. In the presence of caffeine, eyes are severely reduced in size and ommatidia are fused and greatly reduced in number (compare A and B). Staining with elav antibody reveals neuronal differentiation still occurs. Discs are smaller at third instar compared to heterozygous controls (compare C and D) but individual cells are not reduced in size (compare E and F).

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Chapter 5:

Conclusion

Genomes are under constant bombardment by environmental agents, such as ultraviolet (UV) radiation, ionizing radiation (IR) and chemical mutagens. They are even subject to stresses imposed by toxic by-products of normal cellular processes, such as radical oxygen species. In order to maintain genomic integrity, all eukaryotes have evolved signaling cascades called checkpoints for monitoring genetic da. Despite the seeming radical diversities in morphology between various eukaryotes, these checkpoints are well conserved and structurally and functionally similar (Nurse 1990).

I have presented three studies that examine checkpoint functions in the model organism Drosophila. These studies include the development of a novel screening method for generating and isolating mutants whose eye development is susceptible to the cell cycle disrupting drugs hydroxyurea and caffeine; the generation and isolation of mutants for Drosophila *ATM*, a gene whose human homologue is a member of the DNA damage checkpoints; and further characterization of the G2/M and S/M DNA replication checkpoint using various DNA damage and chemotherapeutic drugs. Implications of each of these studies have already been discussed individually, however further observations can be made when they are examined collectively.

There is evidence for the ability of functional checkpoints to compensate for the functions of impaired ones. In mammalian systems, ATM is the key mediator of the responses to DNA damaged by ionizing radiation (IR) or radiomimetic drugs while ATR responds to incomplete replication or DNA damaged by ultraviolet light (UV). However, in the absence of ATM activity, ATR is capable of mediating normally ATM-dependent responses. When ATM-deficient cells are exposed to IR during G1, ATR phosphorylates

and activates p53 in a delayed but discernible manner (Siliciano *et al.* 1997; Banin *et al.* 1998; Canman *et al.* 1998). In addition, ATR function is required to maintain p53 phosphorylation in ATM-positive cells that have incurred high levels of IR-mediated damage (Tibbetts *et al.* 1999). We have seen evidence for a similar role for Drosophila mei-41: we have confirmed previous results that demonstrate *mei-41* mutants are sensitive to the radiomimetic drug methyl methanosulfonate (MMS) and we have quantified this sensitivity. Drawing parallels between mammals and Drosophila, the dramatic sensitivity of *mei-41* mutants may be explained by the supposition that in the absence of *mei-41*, p53 phosphorylation cannot be maintained in response to extensive DNA damage.

ATM and ATR also function synergistically in the intra-S phase checkpoint. ATR and Chk1 normally execute an S phase arrest in response to UV damage while ATM is responsible for S phase arrest in response to IR damage. However, abrogation of ATR function leads to radio-resistant DNA synthesis (RDS), implicating a role for ATR following exposure to IR (Cliby *et al.* 1998). If this role involves Chk1, we expect that Chk1-deficient cells would also undergo RDS and that *grapes* (*grp*) mutants, therefore, would be sensitive to MMS. Indeed, *grp* mutants are sensitive to MMS. Additionally, if mei-41 and grp do behave in a manner analogous to ATR and Chk1, then it is not surprising that *mei-41* mutants would exhibit ten-fold sensitivity compared to *grp* mutants since *mei-41* participates more actively in the responses to MMS.

Given the synergistic responses that ATM and ATR are proposed to play in both the G1 and S phase checkpoints, it would be interesting to see if a combination of haploinsufficiency of *dATM* and absence of *mei-41* or *grp* results in increased sensitivity to MMS. Haploinsufficiency of *ATM* in mice results in increased sensitivity to IR and there are reports that human carriers of *ATM* truncation mutations are more susceptible to some types of cancers (Barlow *et al.* 1999; Bay *et al.* 1999). It would also be interesting

to see if there is a synergistic effect in the responses of ATR and ATM to ongoing replication. If dATM has the ability to compensate for the absence of mei-41, we might expect that haploinsufficiency of *dATM* would increase *mei-41/grp/Dwee1* mutant sensitivity to drugs that interfere with DNA replication, including hydroxyurea, cisplatin and ara-C.

These compensatory functions were the rationale for using caffeine in screening for checkpoint mutants. The first two studies were based on the assumption that inhibition of the ATM and ATR kinases using caffeine would make phenotypes for other checkpoint mutants more apparent (Hall-Jackson *et al.* 1999; Sarkaria *et al.* 1999; Moser *et al.* 2000). For example, this inhibition would preclude the ability of functional mei-41 to compensate for the loss of function of parallel checkpoint components such as those initiated by *dATM*. As was mentioned in chapter 1, we have not yet been able to demonstrate conclusively that the caffeine-dependent *huc* mutant phenotypes arise from checkpoint defects. However, evidence for the validity of this rationale is seen in chapter 2, in which exposure to caffeine does increase *dATM* mutant susceptibility to lethality. Further evidence comes from preliminary data not included in these studies that indicates *Dwee1* mutant hemizygotes and *grp* mutant homozygotes are susceptible to caffeine treatment as assessed by methods similar to those in chapter 3.

Very few screens have been conducted to look for novel checkpoint mutants based on sensitivity to caffeine. While the screening method that resulted in the isolation of the *huc* mutants did not prove to be as straightforward nor as fruitful as we had hoped, perhaps it will instigate the investigation of checkpoint function in Drosophila based on caffeine sensitivity.

Finally, it would be interesting to test for genetic interactions between the *huc* mutants and the *dATM* mutants. For example, an inability of otherwise viable $huc^{95D\text{-}E}$ mutants to tolerate haploinsufficiency of *dATM* would be another line of evidence that

implicates *huc*$^{95D\text{-}E}$ for involvement in checkpoint responses and, again, would confirm the validity of our approach.

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